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THE MORPHOLOGY OF EARLY ATHEROSCLEROTIC LESIONS OF THE AORTA DEMONSTRATED BY THE SURFACE TECHNIQUE IN RABBITS FED CHOLESTEROL

TOGETHER WITH A DESCRIPTION OF THE ANATOMY OF THE INTIMA OF THE RABBIT'S AORTA AND THE "SPONTANEOUS" LESIONS WHICH OCCUR IN IT *

G. LYMAN DUFF, M.D.†; G. C. MCMILLAN, M.D., and A. C. RITCHIE, M.B., Ch.B.

From the Department of Pathology, Pathological Institute, McGill University, Montreal, Que.

The earliest atherosclerotic lesions of the aorta demonstrable by the usual histologic technique of sectioning and staining in rabbits fed cholesterol consist of minute, focal, lenticular collections of large, fat-filled cells which lie immediately under intact endothelium. A small amount of what seems to be extracellular fat is often seen in relation to the fat-filled cells. Endothelial cells rarely contain lipid, and then only in small quantity.

These early lesions are very small, consisting of as few as a dozen fat-filled cells in a section 5 μ thick cut perpendicular to the endothelium, yet they must have been present ante mortem for at least a short time, for time was required for the cells to fill with fat and to become grouped together. Nevertheless, in spite of extensive study, the conventional methods of sectioning have failed to demonstrate earlier lesions. Nor is there agreement as to the probable character of the earlier lesions, and various incompatible theories have been proposed.[1-5]

In these circumstances, it became desirable to study early atherosclerotic lesions by other means. The technique of surface examination of whole mounts of intact aortic intima described by Lautsch, McMillan, and Duff[6] proved well adapted to the problem. This paper will describe early atherosclerotic lesions demonstrated by this technique in the aortas of rabbits fed cholesterol. Because the microscopic

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appearance of a surface preparation is very different from that of the usual histologic section, an account will be given of the anatomy of the endothelium, the intima, and internal elastic membrane of the rabbit's aorta as revealed by the surface technique. The structure of the various kinds of "spontaneous" aortic lesion demonstrated by it will be described also.

Materials and Methods

Approximately 100 untreated New Zealand white rabbits and approximately 250 fed cholesterol were studied. Both males and females were examined. Most of the rabbits were from 10 weeks to 4 months old and weighed 2 to 3 kg. A few were 2 or 3 years old. Some were used to develop technical methods, others for various experiments.

All rabbits were given water ad libitum and were fed Miracle Rabbit Food (Ogilvie Flour Mills). Rabbits on the cholesterol diet were given daily 93 gm. of the rabbit food coated with 6 gm. of corn oil (Mazola oil) and 1 gm. of powdered cholesterol (British Drug Houses). They did not always eat the whole daily ration. All rabbits fed cholesterol remained on the cholesterol diet until death. Twelve rabbits were given corn oil with their food but no cholesterol. Untreated rabbits received daily 93 gm. of the unmodified rabbit food.

The rabbits were sacrificed by an intravenous injection of pentobarbital sodium (Abbott). A massive dose of heparin sometimes was given with the pentobarbital. In most cases, the whole aorta was prepared by the surface technique, but occasionally a portion was embedded, sectioned, and stained in the orthodox manner and studied by longitudinal, transverse, or tangential sections. The surface technique has been described in detail by Lautsch, McMillan, and Duff.[6] Briefly, it is a method whereby the aortic intima, with a little subjacent media, can be stained and mounted on glass slides with the endothelial surface uppermost. The depth of medial staining varies, but rarely are more than one or two layers of medial cells visible (Fig. 1). Microscopic objectives of the highest powers can be used, and the preparations are sufficiently transparent to allow the observer to study easily in three dimensions the intima and any lesions in it. It should be realized, however, that cellular detail is not so clear as in an ordinary section cut at 5 μ. Details of nuclear structure, for example, are hard to resolve. Although other staining methods described by Lautsch, McMillan, and Duff[6] were used occasionally, the surface preparations were usually stained with hematoxylin and Sudan IV, with or without silver impregnation to demonstrate endothelial cement lines.

The staining technique now used differs slightly from that described previously.[6] The aorta, tied to its frame, is removed from the rabbit in one piece. It is kept immersed in warm, physiologic saline solution while it is opened and the adventitial fat is removed. The steps of the staining procedure are:

1. Immerse in 5% dextrose for 5 to 10 minutes.
2. Place the aorta over a suitable dish and, using a chemically clean pipette, cover the endothelial surface with a 0.25% solution of silver nitrate in water. Replace the silver nitrate continuously, tilting the aorta so that the solution runs off to be replaced with fresh silver nitrate from the pipette. Keep the silver nitrate in contact with the surface for 30 seconds. If the solution is left on the aorta longer, excessive precipitate may form.
3. Rinse gently in 5% dextrose.
4. Immerse the aorta in clean dextrose, and expose it to ultraviolet light. Using a 275 watt General Electric Sun Lamp 2 ft. from the aorta, the time of exposure varies from 3 to 15 or more minutes. It is important to control the exposure by examining the aorta microscopically, terminating it as soon as the first, faint cement lines can be seen. Too long an exposure causes over-thick cement lines and too much precipitation.
5. Immerse the aorta in 10% formalin and fix in the dark for 12 to 24 hours.
6. Wash in gently running tap water for at least 1½ hours. The stream should not impinge directly on the endothelium.
7. Immerse in 70% ethyl alcohol for 10 minutes.
8. Cover the endothelial surface of the aorta with a newly filtered, saturated solution of Sudan IV in equal parts of 70% ethyl alcohol and acetone. Leave the stain in contact for 45 seconds, tilting the aorta and replacing the stain as in step 2.
9. Rinse briefly in 70% ethyl alcohol.
10. Wash in gently running tap water for 5 to 10 minutes.
11. Cover the endothelial surface of the aorta with a newly filtered solution of Harris' hematoxylin, tilting the aorta and replacing the stain as in step 2. The hematoxylin is usually left in contact for about 45 seconds, but it is essential to control the staining by microscopic observation after washing. Overstaining renders the preparation too opaque.
12. Dip in acid water (1 part glacial acetic acid in 500 parts of distilled water).
13. Wash in running tap water for 5 to 10 minutes.
14. Rinse in distilled water.
15. Immerse in anhydrous glycerol (98 to 100%) for 48 to 72 hours.
16. Strip off the media as described in the earlier paper.[6]
17. Divide the aorta into sections of convenient length, and mount as described in the earlier paper.[6]

Anatomy of the Aortic Endothelium, Intima, and Internal Elastic Membrane

The study of the intima and its cells is difficult in conventional sections, but becomes relatively easy in surface preparations. In the rabbit, the aortic intima is lined internally by endothelium and limited externally by the internal elastic membrane. Between lies a meshwork of loose connective tissue. The intima is three or four cells thick in the arch, but grows thinner distally until in the abdominal aorta the endo-

thelium often is separated from the internal elastic membrane only by a thin layer of acellular material. Cells of four kinds have been identified in the intima of the rabbit's aorta: endothelial cells, fibrocytes, histiocytes, and what will be called monocytoid cells. Smooth muscle cells can be seen in the media, but none could be identified in the intima. The methods used do not demonstrate mast cells. No capillaries are present in the intima of the rabbit's aorta.

The endothelium of the rabbit's aorta is composed of a single layer of polygonal cells of uniform size, shape, and orientation (Fig. 2). The actual shape of endothelial cells seen in surface preparations depends in part on the direction and the extent of the stretching to which the aorta is subjected before it is fixed. It is, therefore, impossible to be sure of the shape of these cells during life, but probably they are elongated in the long axis of the aorta and about two and a half times as long as wide. Each endothelial cell forms a thin plate, being slightly thickened in the center to contain a flattened nucleus, but tapering to very thin margins. Each cell contains a single nucleus. No multinucleated or anucleated forms such as have been described in other species have been observed in the rabbit. The flat endothelial nuclei are large and oval. Their long axes lie in the long axes of the cells. The nuclear membrane is delicate, the chromatin finely granular. Nucleoli have not been identified with certainty. A rare mitotic figure has been seen. The cytoplasm of endothelial cells often appears vacuolated in surface preparations. The intercellular cement lines between adjacent endothelial cells are delicate, argyrophilic, wire-like structures. Their dimensions, as judged after silver impregnation, can be made to vary widely by varying the technique of impregnation. The "stigmata" and "stomata" described by various writers[7] are rarely seen in surface preparations of the rabbit's aorta. Both are considered to be artifacts, as is the granular material described by McGovern,[8] which is rarely prominent in our preparations.

The fibrocyte is the most common cell in the aortic intima of the rabbit (Fig. 3). Typically, these cells are long, slender, and cigar-shaped. Less commonly, they have many branches, tending to assume a stellate form. From the tips of the elongated cells and from the ends of the stellate branches, fine processes extend for long distances. The nuclei of fibrocytes are large and ovoid, occasionally indented or folded, and in the elongated cells often rather square-ended. Nucleoli can sometimes be identified. Mitosis of these cells has not been seen in the normal intima. The cytoplasm does not contain vacuoles except

that there is often a single vacuole at one or both poles of the nucleus in the elongated cells. In the intima of the arch of the aorta, fibrocytes are numerous and arranged in a somewhat disorderly manner. They form two or three ill-defined layers and tend to lie transversely. More distally, as intimal cells become fewer, the fibrocytes form a single, often incomplete layer and are arranged in a much more orderly fashion, lying transversely so as to form palisades or isolated, sheaf-like groups (Fig. 4).

Typically, the histiocyte is a large cell with abundant cytoplasm. It is usually irregular in shape with many processes (Figs. 5 and 6), but occasionally is rounded with few processes. The processes are very long and become very fine, weaving through the intima to interlace with the processes of other histiocytes and those of fibrocytes. They may branch. The nuclei of histiocytes are similar to those of fibrocytes, though more often reniform, but sometimes contain slightly coarser, more darkly staining chromatin. Nucleoli have not been seen. The cytoplasm is usually homogeneous, but occasionally contains a few vacuoles or granules. Mitotic figures have not been observed in normal intima. Differentiation of histiocytes from fibrocytes is usually easy. The latter are generally long and slender with little cytoplasm; the former are irregularly shaped or rounded with a great deal of cytoplasm. The square-ended nuclei of some of the fibrocytes are distinctive. However, forms of intermediate structure exist, and in every aorta there are a few cells which cannot be classified with certainty, thus precluding an accurate estimate of the relative numbers of histiocytes and fibrocytes. All that can be said is that histiocytes are less numerous and are found singly, scattered here and there among the fibrocytes. In general, they are more numerous in the intima of the arch than in the intima of the abdominal portion of the aorta.

Monocytoid cells are uncommon in the aortic intima of the normal rabbit. These cells are usually round, with only a thin rim of cytoplasm (Fig. 7). In size and structure they closely resemble the large lymphocytes and monocytes of the blood. Their nuclei are round or reniform with a well marked nuclear membrane and heavily staining, coarse chromatin. The chromatin sometimes forms clumps, but nucleoli have not been identified. Mitotic figures have not been observed in normal intima. The rim of cytoplasm is sharply delimited and homogeneous. Rarely, in the normal intima are these monocytoid cells larger, with more abundant cytoplasm and coarse processes like pseudopodia. In these larger forms, both cell and nucleus are often markedly irregular

in shape, and the cytoplasm is often vacuolated (Fig. 8). Monocytoid cells occur singly in the intima, and almost always lie immediately under the endothelium. They are scattered very sparsely, and the whole of a normal aortic intima may contain less than a dozen. They are perhaps more common in areas where the intima is thicker.

The internal elastic membrane of the rabbit's aorta is a thin, fenestrated sheet made up of a dense felt-work of very fine fibers which, for the most part, run in the long axis of the aorta (Fig. 9). In the membrane are many small, round, or oval openings whose long axes lie in the long axis of the aorta. From the intimal surface of the internal elastic membrane, a plexus of coarse elastic fibers extends into the intima. These fibers may branch as they run through the intima to rejoin the internal elastic membrane or to anastomose with other fibers. In general, they run in the long axis of the aorta, though many filaments lie more or less transversely. They often cross the openings in the internal elastic membrane, dividing them as mullions and transoms do windows. Small, delicate, subsidiary elastic membranes may also be found in the intima. These sometimes anastomose with the internal elastic membrane, sometimes with the coarse elastic plexus. The fibers connecting the external surface of the internal elastic membrane to the elastic membranes of the media are not clearly shown in surface preparations.

Immediately caudal to most arterial branches, the aortic intima is thickened so as to make a crescentic mound with its concavity cephalad (Fig. 10). The mounds are thickest where they overhang the caudal edge of the branch, but merge gradually with the surrounding intima as they curve away on either side. They are made mainly of fibrocytes, with a few histiocytes, and are covered by unremarkable endothelium. The only arteries without these caudal mounds are occasional, very small vessels arising proximal to the renal arteries, and the obliterated ductus arteriosus which has a similar mound, but on its cephalic side with the concavity caudad.

Occasionally a monocytoid cell is found on the luminal surface of the endothelium. Less commonly, an irregular, fibrillar mass of fibrin with enmeshed cells similar to those of the blood may lie on intact and apparently normal endothelium. Still less commonly, red blood cells may be scattered over portions of the endothelium. The frequency with which these appearances are observed seems to vary inversely with the thoroughness with which the endothelial surface of the aorta is washed during its preparation.

It is important to note that in the rabbit the normal aortic intima

contains no fat or other material which can be stained with Sudan IV, except a very rare droplet of fat in a fibrocyte or histiocyte.

In summary, the aortic intima of the rabbit is a thin, tapering membrane of loose connective tissue, limited on one side by the fenestrated internal elastic membrane and covered on the other by endothelium. Only rarely does it contain any material stainable with Sudan IV.

"Spontaneous" Lesions of the Rabbit's Aorta

Three kinds of focal, pathologic change were observed in the aortas of untreated rabbits. These lesions will be described to avoid confusion with those found in rabbits fed cholesterol.

The rarest "spontaneous" lesion, seen only once, was a focus of acute inflammation in the intima and media with exudation of many polymorphonuclear leukocytes.

By far the most common "spontaneous" lesion is the medial degeneration first described as chronic endo-aortitis deformans by Israel[9] and subsequently reported by several other writers.[10,11] This change was observed in 50 of 99 rabbits studied by the surface technique in which particular attention was paid to it. The severity of the change varied widely. In the most advanced case, the aorta was converted into an ectatic, calcified tube with irregular, fusiform dilatations. Such severe lesions were rare. More often the lesions were small and multiple. Though they could sometimes be seen from the endothelial surface of the aorta by the naked eye, appearing as rounded, white areas, often with a depressed center and calcification, the majority of "spontaneous" medial lesions were microscopic. The lesions were most common in the ascending aorta and the arch, though those of microscopic size were often found in the cephalic lip of intercostal arteries (Fig. 10). "Spontaneous" medial degeneration was more common in the old rabbits than in the young. As seen microscopically in surface preparations, the lesions were crateriform (Fig. 11). The endothelium was intact, and did not appear abnormal. The walls of the crater were intimal thickenings made up of fibrocytes with a few histiocytes. The base of the crater consisted of a confusion of monocytoid cells, histiocytes, fibrocytes, and smooth muscle cells. All lesions extended a variable distance into the media whose elastic laminae and muscularis were destroyed or disorganized. Around the larger lesions, variable numbers of monocytoid cells lay close beneath the endothelium of the wall of the crater and the surrounding intima. The monocytoid cells in and about the lesions were sometimes round with little cytoplasm, but often were larger, with irregularly shaped, usually vacuolated cytoplasm.

Rarely a monocytoid cell in one of the areas of medial degeneration contained a few, small fat droplets stainable with Sudan IV. Mucoid degeneration or calcification was occasionally seen. Nothing was found to suggest the etiology of "spontaneous" medial degeneration.[11]

The third type of "spontaneous" lesion is uncommon. Occasionally, a clump of from 2 to 100 monocytoid cells was found immediately under the endothelium. The cells might be round, resembling the monocytes or lymphocytes of the blood, or have moderate amounts of cytoplasm, resembling macrophages. They were usually packed closely together. The underlying fibrocytes and the overlying endothelium appeared intact, though the larger lesions bulged into the aortic lumen. Lesions of this type did not contain fat stainable with Sudan IV.

"Spontaneous" atherosclerotic lesions similar to those reported by Bragdon[12] were not recognized. It should be noted, however, that the surface technique as applied in these studies only permits examination of the luminal third of the intramural part of the arteries arising from the aorta, and that in a minority of rabbits the origin of the great vessels of the arch was not included in the surface preparation.

Early Atherosclerotic Lesions in Rabbits Fed Cholesterol

The minimal duration of cholesterol feeding necessary to induce atherosclerotic lesions demonstrable by the surface technique varies very much from rabbit to rabbit and from experiment to experiment. In different experiments, atherosclerotic lesions were found in 6 of 8, 6 of 6, and 1 of 7 rabbits fed cholesterol for from 16 to 48 hours. Atherosclerotic lesions were discovered in a rabbit given access to cholesterol-containing food only 4 hours before its death, but even after 30 days on cholesterol some rabbits remained without lesions.

There is only poor correlation between the duration of cholesterol feeding and the type of atherosclerotic lesion produced. Although rabbits fed cholesterol for a longer period tend to have larger and more numerous lesions, some fed for 1 or 2 weeks have but an occasional, small lesion while others fed for only 24 hours may show well developed lesions with foam cells. Moreover, aortas with large lesions often contain also small lesions identical with those seen in aortas having only small lesions.

The atherosclerotic lesions demonstrated by the surface technique were always focal. The types of small atherosclerotic lesion and a typical large one will be described. It should be realized, however, that intermediate forms occur, so that division into small and large lesions is arbitrary.

In the usual type of small atherosclerotic lesion there was no increase in the number of intimal cells, and no disorganization or modification of their arrangement. The only abnormality detected was the presence in the intima of droplets of fat which stained with Sudan IV. These droplets varied in size from the limit of resolution of an objective of the highest power to 2 μ in diameter. For the most part, they were in fibrocytes, forming botryoid clumps at the poles of the nuclei (Fig. 12). They were also in histiocytes, situated in the main perinuclear mass of cytoplasm or in the fine processes. In any one cell, the droplets were usually few in number, and did not distort its shape. It is not clear whether extracellular fat was present. Small droplets of fat were seen both singly and in clumps, apparently at some distance from a cell, but it was often possible to demonstrate that these were contained in one of the long, ramifying processes of a histiocyte. The methods available are not sufficiently reliable to determine whether this is always the case. The size of these small lesions varied. At one extreme, the only fat demonstrable in the whole aortic intima was contained in a single fibrocyte. More commonly in small lesions, ten to fifty fibrocytes and a few histiocytes contained fat. Rarely, the lesions were quite extensive, and in one rabbit extended over an area of intima 2 by 3 mm. In some lesions, most of the fibrocytes and histiocytes in the center of the lesion contained droplets which stained with Sudan IV, while peripherally the proportion of fat-containing cells gradually fell to zero. In others, fat was found in only occasional fibrocytes which lay scattered among those without fat. The lesions may be found in any part of the aortic intima, though they were more common in its thicker parts, the arch and the arterial mounds. The endothelium overlying the lesions and the media beneath did not appear abnormal.

Much less commonly, small lesions of a different type were seen. Lying immediately under intact endothelium, were from five to twenty monocytoid cells, a few of which contained fat droplets. The monocytoid cells were usually irregularly shaped with a moderate quantity of cytoplasm. Occasionally, one or two of the underlying fibrocytes or histiocytes also contained droplets of fat. Small lesions of this type were not sufficiently common to allow any conclusion as to their distribution. Even more rarely, one to three isolated monocytoid cells filled with fat were seen immediately under the endothelium in an otherwise unremarkable area of intima (Fig. 13).

In larger lesions, the number of intimal cells was always increased. Monocytoid cells and histiocytes accumulated in the center of the lesion, forming a moderately well demarcated core (Fig. 14). Probably fibrocytes increased in number little, if at all, though it is hard to be sure

because they were often obscured by the great numbers of histiocytes and monocytoid cells. The amount of fat demonstrable by Sudan IV tended to become greater as the number of intimal cells increased. Not only were there more fat-containing cells, but individual cells contained more fat. The fat droplets tended to be larger, sometimes reaching a diameter of 5 μ. Some were anisotropic (Fig. 16). Cells which contained much fat were usually larger and less branched, resembling more and more closely the typical globular, fat-distended "foam cells" (Fig. 15). In the deeper part of the lesion, histiocytes were predominant, and all forms intermediate between a typical, fat-free histiocyte and a foam cell were found. Monocytoid cells were most common near the endothelium, where they were often round and relatively small, with only a thin rim of cytoplasm and no fat droplets. However, intermixed with the rounded forms were larger monocytoid cells, with variable quantities of irregularly shaped cytoplasm. These larger forms extended more deeply, to lie among the histiocytes. They sometimes contained fat droplets which stained with Sudan IV, or vacuoles which did not take the Sudan stain. Those containing large quantities of fat tended to be globular and so resemble fat-filled histiocytes. Indeed, it was usually impossible to determine whether a particular fat-filled cell was a histiocyte or a monocytoid cell. Forms intermediate between a foam cell and a monocytoid cell were observed just as were forms intermediate between a foam cell and a histiocyte. The monocytoid cells and histiocytes were usually clumped closely together with very little ground substance between them, so that it was impossible to determine whether extracellular fat was present. The great bulk of the fat was certainly intracellular. Surrounding the central core in which the number of intimal cells was increased was a zone in which the intimal cells retained their normal number and arrangement, but in which some fibrocytes and occasional histiocytes contained fat. The proportion of fat-containing cells in the peripheral zone varied, though individual cells rarely contained more than a few, small droplets. The zone varied from several times the diameter of the central core to a quite narrow width. The endothelium overlying the lesions and the media beneath remained unremarkable. In rabbits fed cholesterol, fat was rarely seen in endothelial cells, and then only in minute quantity. The larger lesions in which the number of intimal cells was increased could be found in any part of the aorta, but like the smaller ones they were more common in the parts where the intima was thick, i.e., the arch and the arterial mounds. They varied in diameter from 200 μ to 1 cm., and contained from a few histiocytes and monocytoid cells to several hundred.

It must be emphasized that there was considerable variation in structure from lesion to lesion. There might be a large quantity of fat scattered in fibrocytes but little or no increase in the number of intimal cells. Or only a moderate quantity of fat might be demonstrable in a considerable accumulation of histiocytes and monocytoid cells. The ratio of histiocytes to monocytoid cells varied within wide limits. There might be only four or five histiocytes and monocytoid cells in a lesion and yet all were distended to form foam cells; or there might be many cells but none with enough fat to warrant the name of foam cell. In some lesions, almost all of the fat was in fibrocytes; in others, a great proportion was in histiocytes or monocytoid cells.

Mitotic figures were rarely seen in surface preparations of atherosclerotic lesions, even when the increase in the number of intimal cells was great. During preparation by the surface technique, 1 or 2 hours could elapse between the death of the rabbit and the fixation of the aorta. During much of this period, the aorta was kept warm in physiologic saline solution or 5 per cent glucose. It is possible that mitotic figures present at the time of death might have come to completion during this interval before fixation, and so the fixed preparation would show a mitotic rate lower than that present during life. To reduce this chance, several aortas were perfused with large quantities of 10 per cent formol-saline solution immediately before and during the death of the rabbits. Mitotic figures were not found to be more numerous in the aortas prepared in this way. Nuclear forms similar to those of amitotic division were seen, but not commonly.

In rabbits fed cholesterol, the number of scattered monocytoid cells in the intima was sometimes increased five to twenty times. The cells lay immediately under the endothelium, either singly or in small clumps of two or three. Most of them had little cytoplasm. In one experiment, this increase was seen in 15 of 28 rabbits fed cholesterol less than 3 days, and less commonly in those fed for a longer period. The cells do not contain fat droplets. A similar increase in scattered monocytoid cells was seen also in rabbits given various kinds of intravenous injection.

The number of cells found on the luminal surface of the endothelium of rabbits fed cholesterol varied widely. Often there were none. Sometimes there were a considerable number, sometimes single or clumped monocytoid cells, sometimes blood cells enmeshed in fibrin. For the most part, the luminal cells were scattered in a random manner, lying on apparently normal endothelium and intima. If the intima was torn, or folded, luminal cells or masses of fibrin might be entrapped in the artifact. Rarely, monocytoid cells were found on the endothelium over-

lying atherosclerotic lesions when not present on the endothelium elsewhere. In a few rabbits fed cholesterol for less than 72 hours, a moderate number of monocytoid cells lay on intact endothelium overlying lesions in which the number of intimal monocytoid cells and histiocytes was markedly increased though there was little fat. This appearance was striking but unusual. In no case was a monocytoid cell seen in such a position that it appeared to be passing through the endothelium. In all probability, the number of luminal cells found depended largely on the thoroughness with which the endothelium was washed during its preparation.

In rabbits fed cholesterol, fat was often found in the lesions of "spontaneous" medial degeneration, even though present nowhere else in the aorta. The fat in the "spontaneous" lesions was for the most part close to the endothelium, droplets being found in monocytoid cells of medium size and, to a lesser degree, in histiocytes and fibrocytes. The presence of extracellular fat was again uncertain. If a "spontaneous" lesion contained much fat, fat-containing fibrocytes and histiocytes were found around the lesion, just as fat-containing fibrocytes were found around the larger atherosclerotic lesions. There was again much variation from rabbit to rabbit. Many rabbits with "spontaneous" medial degeneration were fed cholesterol for many days, and yet no fat was demonstrable in the lesions. The presence of "spontaneous" medial lesions did not seem to modify the distribution or localization of atherosclerotic lesions in the aorta.

In summary, the early atherosclerotic lesions demonstrated by the surface technique in the aortic intima of rabbits fed cholesterol were focal. They occurred most frequently in the zones in which the intima was normally thick—the arch and the arterial mounds. The usual type of small lesion consisted of fat-containing fibrocytes and histiocytes, but showed no alteration in the number or arrangement of the intimal cells. In larger lesions, the number of intimal cells was increased by an accumulation of histiocytes and monocytoid cells in the center of the lesion. Some of these cells and some of the surrounding fibrocytes and histiocytes contained fat.

Discussion

By means of the surface technique, the whole aortic intima can be mounted on glass slides with its endothelial surface uppermost, and thus examined microscopically. The normal and morbid structure of the intima are particularly well demonstrated. For the present purpose, however, the chief virtue of the technique is that it allows morphologic study of earlier atherosclerotic lesions than have been described previously. The orthodox histologic methods of sectioning

and staining can rarely demonstrate atherosclerotic lesions of the aorta in rabbits fed cholesterol for less than 4 weeks,[3] but by the surface technique lesions were demonstrated in one rabbit given access to cholesterol-containing food for only 4 hours and were commonly found in rabbits fed cholesterol for 16 hours or more.

If it be assumed that the smaller fatty lesions demonstrated by the surface technique are precursors of the larger, the histogenesis of these early atherosclerotic lesions seems clear. The smallest (earliest) lesion detected consisted of a few intimal cells containing fat droplets. Usually there was no alteration in the number or arrangement of the intimal cells at this stage, the fat being in otherwise normal-appearing fibrocytes and histiocytes. Rarely, the fat was found in subendothelial monocytoid cells. As the lesion grew larger (older), histiocytes and monocytoid cells accumulated in its center, and the quantity of demonstrable fat tended to increase. Not only were there more fat-containing histiocytes and monocytoid cells, but the individual cells contained greater quantities of fat. The lesion also spread peripherally, as a variable proportion of the surrounding fibrocytes and histiocytes came to contain fat droplets. In all these lesions, the fat was mostly, if not entirely, intracellular. No abnormality was detected in the endothelium.

The monocytoid cells probably entered the intima from the blood. The fact that immediately under the endothelium they were often round with little cytoplasm, closely resembling the monocytes and large lymphocytes of the blood, while more deeply they were larger, suggests that monocytes or lymphocytes penetrated the endothelium to enter the intima, and there increased in size, gaining more cytoplasm to assume the larger monocytoid form. This suggestion is in accord with the observation that in the ear chambers of the rabbit, monocytes pass from the blood into the tissue, and there become larger and assume the usual form of a phagocyte.[13] The significance of the moderate numbers of fat-free monocytoid cells sometimes found adherent to the luminal surface of the endothelium is equivocal. Their presence is in accord with the theory that monocytes or lymphocytes from the blood adhere to the endothelium and then crawl through into the intima, but it must be remembered that they were found only occasionally and that they could have become adherent post mortem. The monocytoid cells in the intima may increase in number not only by the arrival of new immigrants but also by mitosis. The relative importance of these two processes is unknown.

In the larger lesions, the number of histiocytes was increased. This increase was probably due mainly to mitosis. Not only was there no

evidence of the immigration of histiocytes from neighboring parts of the intima, but observations on the ear chambers of the rabbit have shown that histiocytes move from their position rarely, if at all.[13] It is possible that the number of histiocytes was augmented also by the transformation of fibrocytes or monocytoid cells. That fibrocytes may be so transformed is improbable. It was not possible to demonstrate any diminution in the numbers of fibrocytes around the lesions, and studies on the ear chambers of the rabbit have failed to demonstrate any transformation of fibrocytes into histiocytes.[13] Monocytoid cells probably are transformed into histiocytes. Intermediate forms could be found among the fat-filled cells of the larger lesions, and in the ear chambers of the rabbit, monocytes have been seen to leave the blood, and after a while become fixed as tissue histiocytes.[13]

It might be maintained that the finding of isolated, fat-filled monocytoid cells scattered immediately under the endothelium of an area of otherwise unremarkable intima gave some support to Leary's theory[2] that the fat of atherosclerotic lesions is carried to the intima by phagocytes, but it should be remembered that such isolated, fat-filled monocytoid cells were rare, and that nothing was seen to suggest that larger lesions developed from them. In the great majority of small lesions, the fat was contained in fibrocytes or histiocytes, not in monocytoid cells. Also, fat droplets were not evident in the monocytoid cells adhering to the luminal aspect of the endothelium. Nor was anything found to support the theory that some of the lesions studied were produced by the incorporation of thrombus, in the manner described by Duguid.[4] The occasional mass of fibrin found on the luminal surface of the endothelium nearly always lay on unaffected intima. It seems more likely that these fibrinous masses resulted from postmortem clotting than that they were attached to the intima during life. Their association with tears in the endothelium and other artifacts supports this conclusion. The failure to find evidence supporting Duguid's theory in these early atherosclerotic lesions of the rabbit does not imply that incorporation of fibrin or thrombus may not occur subsequently during the further development of the lesions. It can, however, be concluded that in these small lesions the fat was not brought by blood-borne phagocytes, and that the incorporation of fibrin played no part in their genesis.

The present studies have done little to show the nature or origin of the fat droplets found in early atherosclerotic lesions. The avidity of the droplets for Sudan IV suggests that they contain much neutral fat, but they may, of course, also contain considerable quantities of other substances. It is possible that in very small lesions, in which only a

few small fat droplets were seen in any one cell, the droplets were due to phanerosis rather than to the accumulation of fat from without the intima. In large lesions, the foam cells contained so much fat that this possibility seems remote. It is therefore probable that even in small lesions the fat of the intracellular droplets reached the intima from outside, that is, from the blood plasma. If this were so, it seems that any or all of three localizing mechanisms might have been operative. It might be that the endothelium overlying the site of a lesion became more permeable so that excessive quantities of fat reached that portion of the intima. Against this possibility is the failure of the present studies to demonstrate any morphologic abnormality in the endothelium overlying the lesions. A second possibility is that the intercellular ground substance at the site of the lesion became changed, so that the fat of the tissue fluid was precipitated, subsequently to be picked up by the fibrocytes and histiocytes. Against this is the lack of any considerable quantity of extracellular fat in the early lesions, and the belief that fibrocytes display little phagocytic activity. The third possibility is that from some fault in their biochemical structure the fibrocytes and histiocytes became unable to deal with fat which was therefore precipitated in their cytoplasm. There is little reason at present to prefer any one, or any combination, of these theories. Nor is it clear why these early lesions formed in one part of the intima rather than another. They were more common in its thicker parts, where there were more fibrocytes and histiocytes, but even in the thick intima of the arch and the arterial mounds only a minority of the cells were involved.

Care has been taken in this paper to avoid expressing any opinion as to whether the monocytoid cells thought to enter the intima from the blood arose from monocytes, lymphocytes, or both. The methods of staining employed in the surface technique were not sufficiently delicate to distinguish with certainty between monocytes and lymphocytes, even in blood films. That monocytes can enter the tissues, forming phagocytes, is generally agreed. There is no agreement that lymphocytes can do so. Some authors have maintained strongly that lymphocytes can be transformed into macrophages in this way, others equally strongly that they cannot. The evidence offered by the present study does not justify our entering the controversy.

Summary

The whole intima of a rabbit's aorta can be mounted on slides with the endothelial surface uppermost and examined by the highest powers of the microscope. The normal structure of the intima of the rabbit's

aorta as revealed by this technique, and the "spontaneous" lesions which occur in it, are described.

By means of this technique, very small atherosclerotic lesions were detected in the aortic intima of rabbits fed cholesterol. The structure of these small lesions is described, and their pathogenesis is discussed.

The work reported here is based in part on the researches of Drs. E. V. Lautsch, A. N. Rota, and Z. Fluss, done under the direction of Drs. G. Lyman Duff and G. C. McMillan and submitted in the form of theses[14-16] to the Faculty of Graduate Studies and Research, McGill University.

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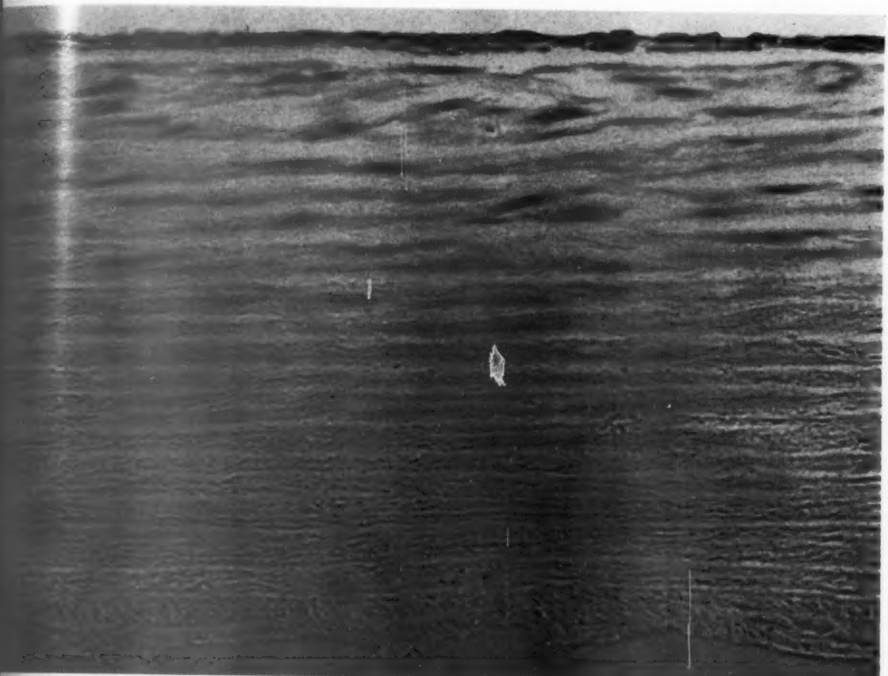
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Cross section of an aorta prepared by the surface technique, to show the thickness of the preparation and the depth to which it is stained. Hematoxylin and silver nitrate stains. × 350.

FIG. 2. Surface preparation which shows the size, shape, and arrangement of the endothelial cells and their nuclei. The vertical striations are caused by the underlying tissues. Hematoxylin and silver nitrate stains. × 400.



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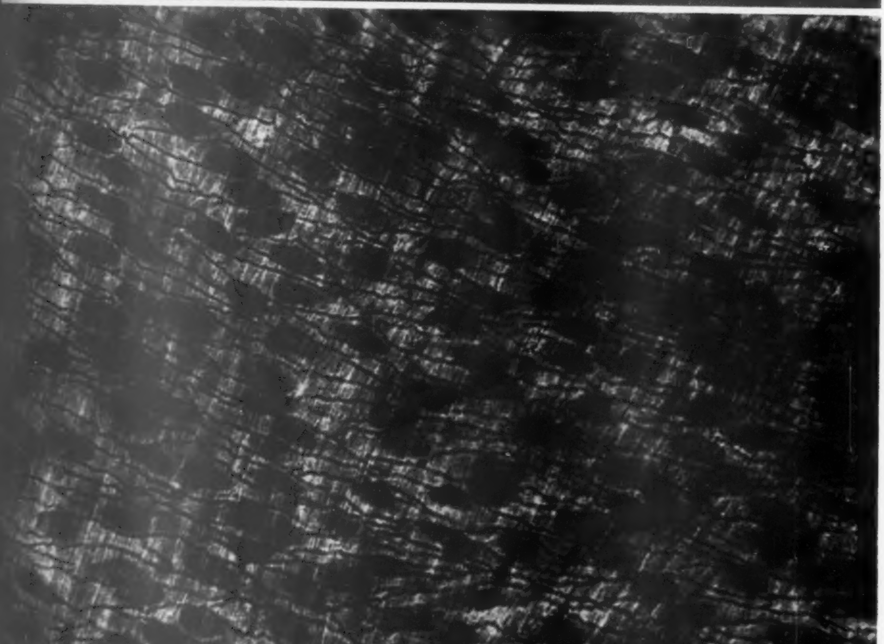

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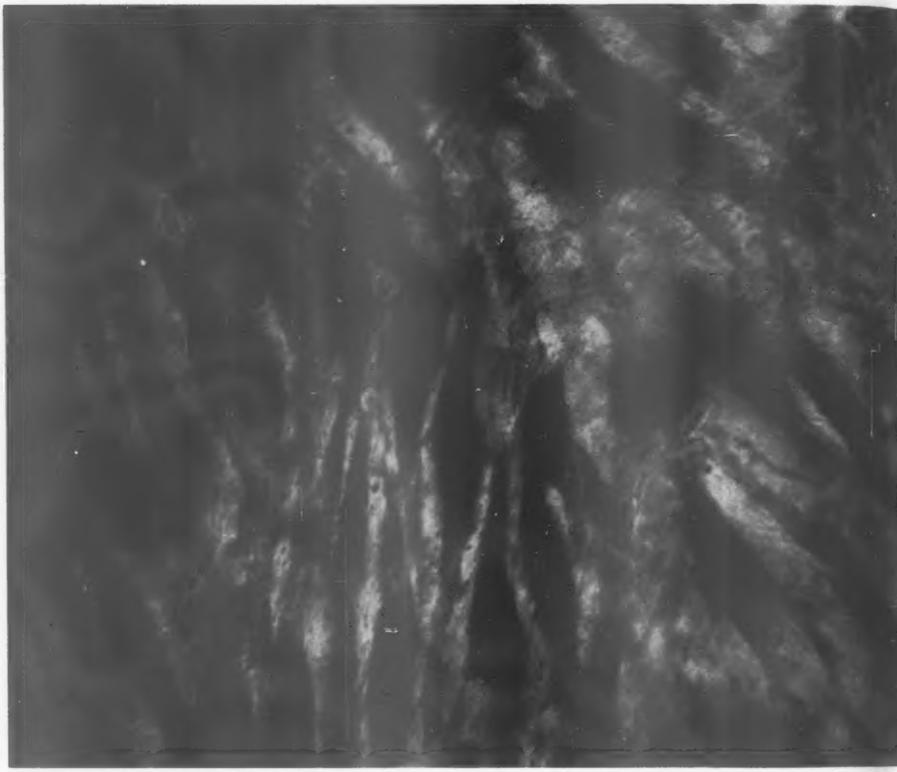


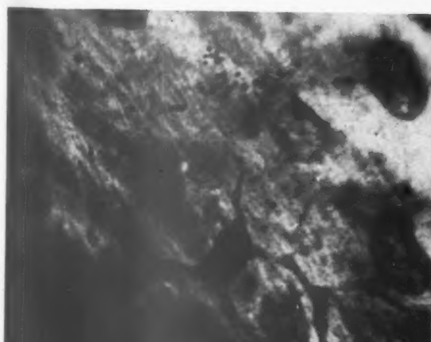
3

FIG. 3. Surface preparation. Fibrocytes in the intima. The cell at the upper right corner is a histiocyte. Hematoxylin, Sudan IV, and silver nitrate stains. × 1,000.

FIG. 4. Surface preparation. Sheaf-like arrangement of fibrocytes in the distal part of the aorta. Hematoxylin, Sudan IV, and silver nitrate stains. × 280.

FIGS. 5 and 6. Surface preparations. Branched histiocytes containing small droplets of fat in rabbits fed cholesterol. Hematoxylin and Sudan IV stains. × 1,000.

4



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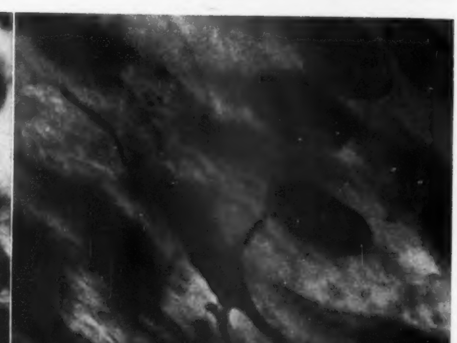

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FIG. 7. Surface preparation. Small monocytoid cell in the intima. Endothelium can be seen, but is out of focus in the center. Hematoxylin, Sudan IV, and silver nitrate stains. × 1,000.

FIG. 8. Surface preparation. Large monocytoid cell. The endothelium which overlay the cell has been folded back. Hematoxylin, Sudan IV, and silver nitrate stains. × 1,000.

FIG. 9. Surface preparation. Fenestrated elastic membrane. Chlorazol black E-chlorazol azurine G stain. × 400. (Reproduced by permission from *Laboratory Investigation.*[6])

FIG. 10. Surface preparation. Crescentic mounds caudal to intercostal arteries. The dark area below the left artery and that above the right artery are lesions of "spontaneous" medial degeneration. Hematoxylin, Sudan IV, and silver nitrate stains. × 30.

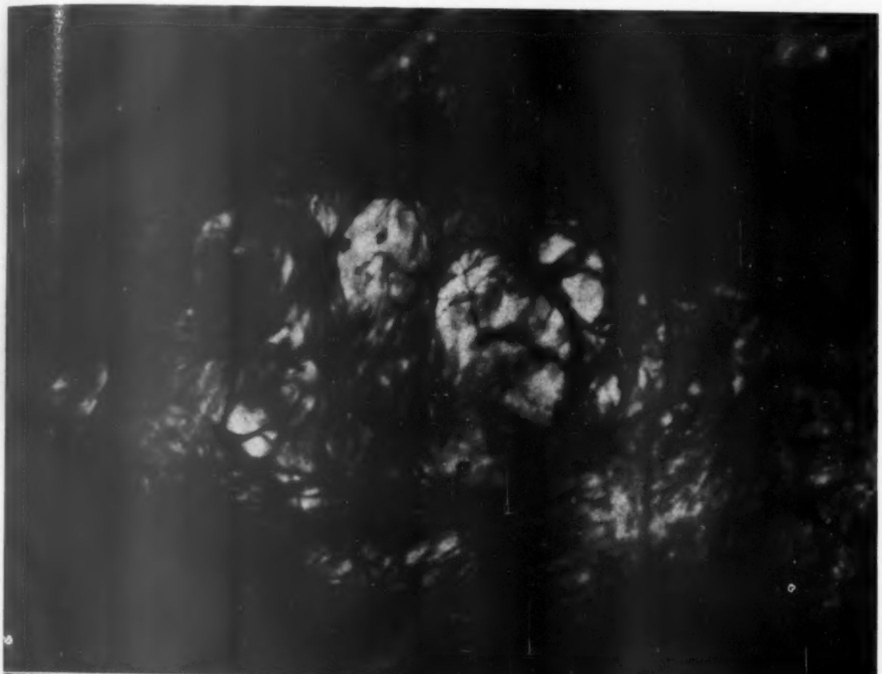

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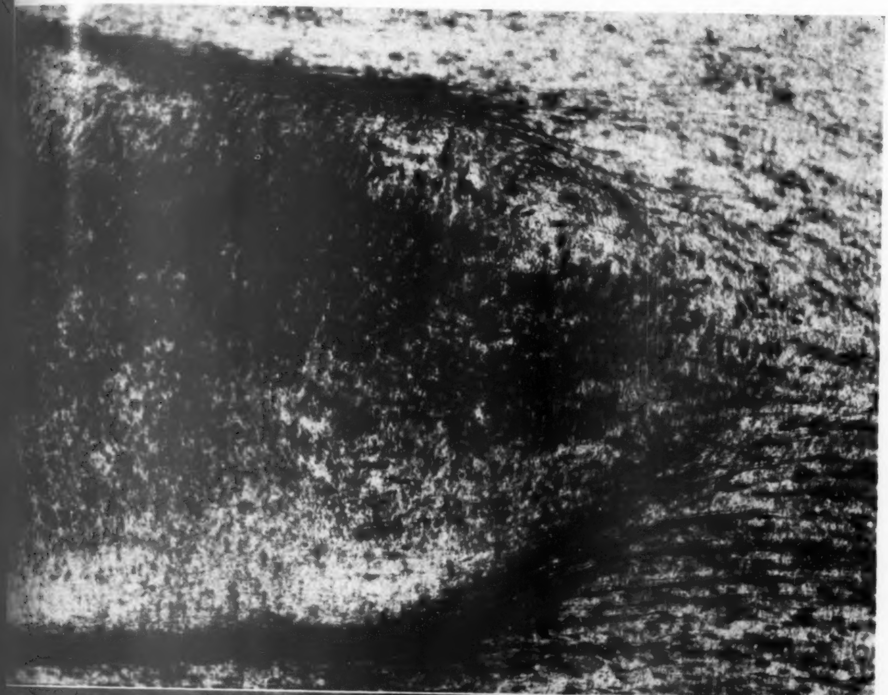
FIG. 11. Surface preparation. Lesion of "spontaneous" medial degeneration, showing crater-like form. Hematoxylin and Sudan IV stains. × 120.

FIG. 12. Surface preparation. Small atherosclerotic lesion in a rabbit fed cholesterol. Fibrocytes contain droplets of fat which appear dark. A histiocyte in the lower left corner shows no fat. Hematoxylin, Sudan IV, and silver nitrate stains. × 1,000.



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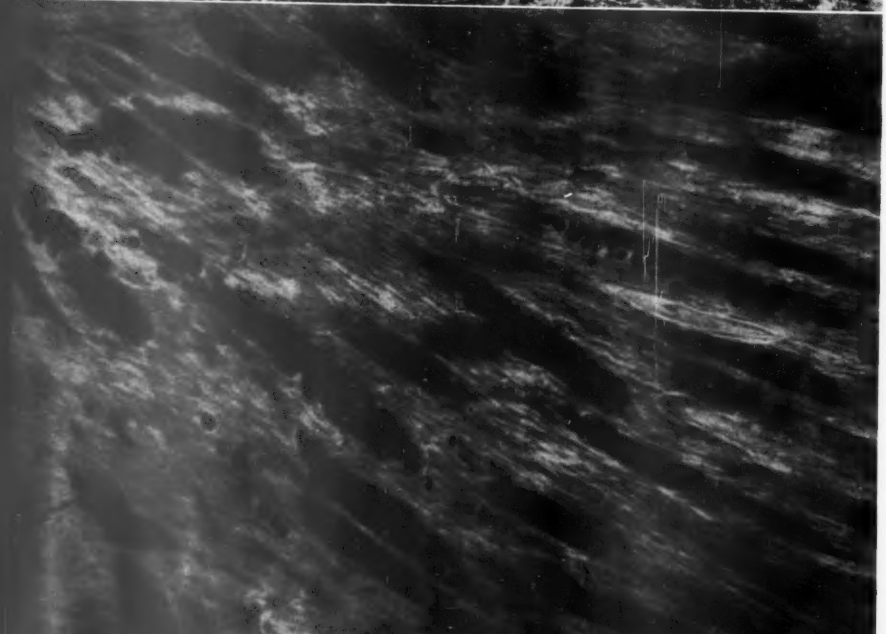

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FIG. 13. Surface preparation. Isolated monocytoid cell containing fat in a rabbit fed cholesterol. Hematoxylin and Sudan IV stains. × 1,000.

FIG. 14. Surface preparation. A larger atherosclerotic lesion in a rabbit fed cholesterol. The number of intimal cells is increased, foam cells, histiocytes, and monocytoid cells being recognizable. Endothelium can be seen at the upper right corner. Hematoxylin, Sudan IV, and silver nitrate stains. × 1,000.

13

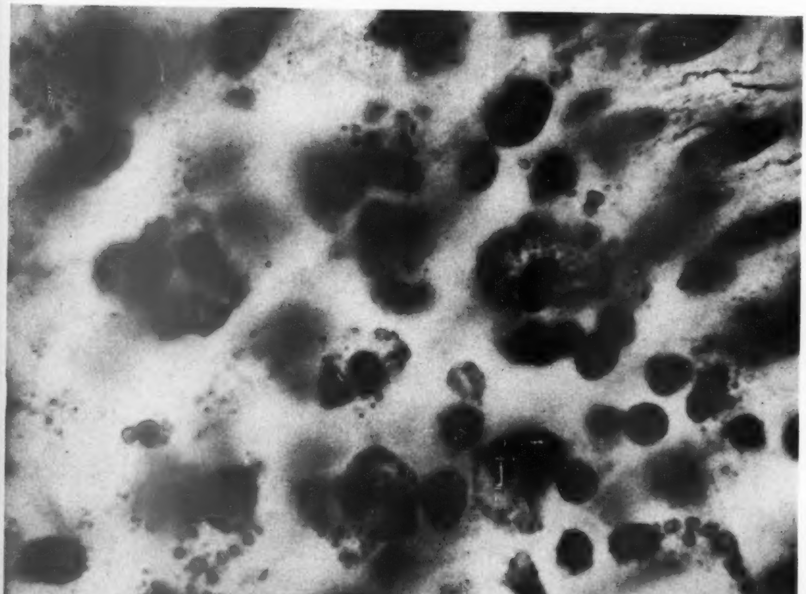

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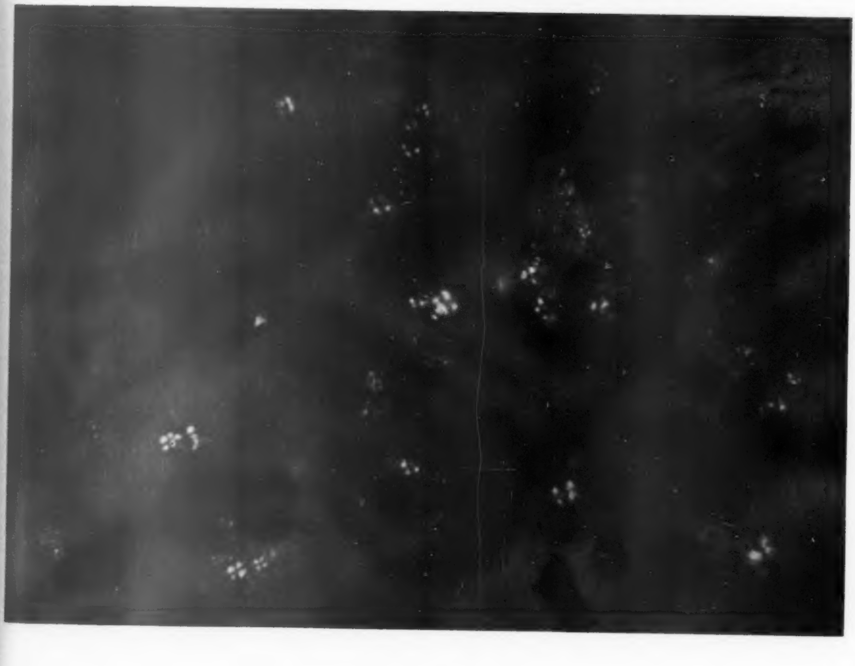
FIG. 15. Surface preparation. Large atherosclerotic lesion in a rabbit fed cholesterol. Foam cells are numerous. Fat-containing fibrocytes and fat-containing histiocytes can be seen. Hematoxylin and Sudan IV stains. × 1,000.

FIG. 16. Surface preparation. Same field as that illustrated in Figure 14 illuminated with polarized light and photographed through a partially crossed polarizing disk. Hematoxylin, Sudan IV, and silver nitrate stains. × 1,000.

15



16

THE QUANTITATIVE APPRAISAL OF ATHEROSCLEROSIS *

IRA GORE, M.D., and CARLOS TEJADA, M.D.

From the Department of Nutrition, Harvard School of Public Health, and the Department of Pathology, Harvard Medical School, Boston, Mass.; the Laboratory Service, Veterans Administration Hospital, West Roxbury, Mass.; and the Institute of Nutrition of Central America and Panama (INCAP), Guatemala City, Guatemala

The customary pathologic grading of atherosclerosis does not portray adequately the prevalence and extent of subclinical vascular disease, although it has permitted satisfactory clinical correlation with occlusive arterial disease. Since it is based upon the experience of the observer, it is poorly adapted to a study by a variety of observers in widely separated localities and under assorted circumstances.[1] Accordingly, it is not surprising that some of our prevalent concepts stem largely from indirect evidence. The low frequency of myocardial infarction among the natives of Japan, or the Bantu of South Africa, is the basis for believing that atherosclerosis is less severe among them. Higginson and Pepler[2] deplored the lack of more precise comparative pathologic data in evaluating the incidence of coronary atherosclerosis among the South African Bantu. Similarly, the reduced frequency of coronary arterial disease under conditions of chronic undernutrition in World War II has been attributed to a reduced prevalence of atherosclerosis.[3,4] Although such conclusions may be correct, direct substantiating pathologic evidence would be more satisfactory and would reduce the possibility of missing unsuspected factors. A more objective procedure of assay in recording necropsy observations of atherosclerosis must be adopted. Practically, the procedure must be easy to apply and must avoid undue demands upon the time and resources of the pathologist. Even the most precise technique would be doomed to failure if its complexity discouraged widespread adoption. The following procedure, devised to compare the prevalence of atherosclerosis in Guatemala, other countries of Central America, and in the United States of America, seems to meet these requirements. Although it will be described as it is applied to the aorta, which is the most readily available vessel and the one most uniformly examined, it is equally applicable to any other vessel. It is limited to gross inspection and requires nothing more than systematic observation, estimation, and recording.

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Technique

Appraisal

In an atherosclerotic aorta, the extent or area of intimal involvement and the character of its lesions are to be considered separately. Extent of disease may be expressed by the use of five groups which correspond roughly with the traditional grades of severity; these are outlined in Table I. In practice, the fractional areas listed parenthetically facilitate visual estimation.

Table I
Extent of Atherosclerosis

Group	Traditional grading	Proportion of intima diseased
O	Negligible	Less than 5% (less than 1/20)
A	Minimal (+)	6–15% (less than 1/6)
B	Mild (+ +)	16–33% (less than 1/3)
C	Moderate (+ + +)	34–50% (less than 1/2)
D	Severe (+ + + +)	More than 50% (more than 1/2)

The character of the intimal lesions observed is the second factor to be considered since grouping does not include this information. In general, atherosclerotic lesions, as listed in Table II, are of four types graded from 1 to 4 in the order of their pathologic importance and also, presumably, in the order of their development.

Table II
Types of Atherosclerotic Lesions

Grade 1: *Lipid streaks, spots, and patches.* These are very superficial, thin, yellow, subendothelial accumulations which may just perceptibly elevate the internal surface. Small, punctate, discrete, yellow, pure lipid, nodular elevations (lipid spots) in the ascending aorta are included in this category (Figs. 1 and 2).

Grade 2: *Elevated, smoothly surfaced, fibrous plaques of variable lipid content.* The pearly white fibrous plaque is the type lesion of this category but others are yellow and distinguishable from grade 1 only by the associated presence of sclerosis (Fig. 3).

Grade 3: Plaques with ulceration, necrosis, or hemorrhage (Fig. 4).

Grade 4: Calcified plaques.

By grouping and grading according to Tables I and II, both the extent of atherosclerosis and its character may be expressed by a simple formula or "atherosclerotic profile." This consists of the group letter followed by a number, each of whose four digits corresponds respectively and sequentially with one of the grades of lesions. In this fashion, the figure representing the proportion of grade 1 lesions would be the first digit while that representing grade 4 lesions would be the

fourth. By expressing the whole of the disease as unity or one, the part each grade contributes to it, and the number representing it in the profile, may be estimated visually in tenths. Each individual figure may vary from 0 to 10, but it is apparent that their sum must always be 10 (10 tenths), unless atherosclerosis is completely absent. For example, a severely diseased aorta with involvement of more than half of its intimal surface would be classed as group D. If it is ascertained that one tenth of the lesions are grade 1, two tenths grade 2, one half grade 3, and two tenths grade 4, the atherosclerotic profile would be D 1252.

Evaluation

For statistical evaluation of large numbers of cases, it would be advantageous to reduce the profile to an "atherosclerotic index." This may be accomplished by assigning weights to the factors involved in the profile. Simple arithmetic weighting based on the area involved is suggested for the groups as listed in Table III, since it seems reasonable to infer that the extent of atherosclerosis is directly related to its clinical importance.

TABLE III
Weighting for Extent of Disease

Group	Area involved	Approximate mean area	Weighting
	%	%	
O	0– 5	2.5	1
A	6– 15	10.0	4
B	16– 33	25.0	10
C	34– 50	40.0	16
D	51–100	75.0	30

On the other hand, a linear relation would not adequately express the difference in clinical significance between lipid streaks and ulcerated plaques. Since the physiologic processes of growth and degeneration have a semi-logarithmic relation, it is proposed that the grades be weighted logarithmically as in Table IV. However, grades 3 and 4 are assigned equal weight since there is no basis for considering that calcified lesions are more important than the ulcerated, necrotic, or hemorrhagic plaques of grade 3.

TABLE IV
Weighting for Grade of Lesion

Grade	Weight
1	1
2	10
3 and 4	100

Utilizing the example cited above, with the profile D 1252, an atherosclerotic index may be derived by the following calculation: 30 (1/10 × 1 + 2/10 × 10 +

$5/10 \times 100 + 2/10 \times 100) = 30 \times 72.1 = 2163$. Since the theoretic range is from 0 to 3,000, the additional introduction of a constant multiple, 1/30, provides a more convenient index ranging from 0 to 100. In the cited example, the final atherosclerotic index, to the nearest whole number, then becomes $2163 \times 1/30$ or 72.

Narrowing Index for Visceral Arteries

The major clinical problems in atherosclerosis arise from occlusive changes in the arteries to the heart, brain, and/or lower extremities. Since they may dilate, narrowing is not an invariable consequence of even severe disease in these vessels. Accordingly, adequate pathologic correlation requires some index of luminal constriction to augment the evaluation represented by the atherosclerotic profile. Fortunately, multiple transection, a common technique for examining the coronary arteries at necropsy, is ideally suited for estimating luminal patency. The impairment of circulation by narrowing, following Poiseuille's principle, is proportional to the cube of the fractional lumen remaining. For example, a coronary artery reduced to one fourth of its usual size can convey only 1/64th of the normal volume; its capacity is reduced 64 fold. This factor then provides the weighting to be applied to each of the four degrees of narrowing (Table V) to derive a "narrowing index."

TABLE V
Luminal Narrowing

Degree of narrowing	Diameter of residual lumen	Significance of weight
0	More than 9/10 of normal	0
1	More than 3/4 of normal	2
2	More than 1/2 of normal	8
3	*Less* than 1/2 of normal (assuming an average of 1/4)	60

Inasmuch as atherosclerosis is characteristically irregular and patchy, multiple foci of narrowing are the rule. Of these only the five most severe are considered. Their sum, when weighted according to Table V, would range from 0 to 300 and must be multiplied by the constant factor, 1/3, to yield an index of narrowing which ranges from 0 in normal, to 100 in maximally diseased vessels.

DISCUSSION

The foregoing assay procedure provides for stepwise changes in a process which is continuously variable. Its application, therefore, is bound to require arbitrary decisions. Nevertheless, experience with it

in more than 1,000 cases has convinced us that it is practical and yields reasonably consistent results. The entire length of the opened aorta is examined from the valve ring to the bifurcation. Omission of the aortic ring may mean missing some of the lesions that Holman's group[5] have found to be a frequent and early manifestation in young individuals. Although many of the grade 1 lipidic lesions may be seen in the fresh or formalin-fixed aorta, there is no doubt that Sudan staining as done by the workers of Louisiana State University[5] increases the quantity detected in the first 2 decades. The discrepancy is not great in adults. Since our immediate goal is to learn the prevalence of gross disease rather than its earliest manifestations, staining is not done as a routine procedure. In any case, data derived from stained aortas are not comparable with figures derived from unstained ones.

Little difficulty is encountered in grouping by area of involvement. Although visual estimation results in considerable overlapping between consecutive groups, statistically such errors cancel each other. Puckering, scarring, and even focal calcification at the site of the obliterated ductus is not considered part of the atherosclerotic process.

In the characterization of the lesions the portion of the disease which is calcified or ulcerated is estimated in tenths. Of this total value, the proportion which is calcified constitutes grade 4 lesions, the remainder are grade 3. As a radiologically detectable feature, the clinical importance of calcification is such that it has been our practice, though it may magnify the situation, to record even a small single focus. Similarly, any ulceration observed means that at least one tenth of the disease is to be considered grade 3. Although mural thrombi are really a complication rather than a part of atherosclerosis, by and large, they originate upon and cover areas of ulceration. Accordingly, they have been classed with grade 3 lesions, acknowledging the impossibility of distinguishing incorporated areas of intact endothelium once organization has appeared. For purposes of record, we have indicated the proportion of such involvement in the entry on the original data sheet.

Frequently, lesions are both calcified and ulcerated. In this event, it has been our policy to tabulate them with grade 3 lesions and indicate the mineralized portion parenthetically under grade 4. The latter figure, of course, does not enter into the calculation of the atherosclerotic index, but does provide a record of the extent to which the aorta is calcified. In the example cited above, if somewhat more than half of the ulcerated lesions were also calcified, the previous profile would be written as D 125(3)2. According to this representation, half of the atherosclerotic lesions are calcified, a parenthetic three tenths, already

counted under grade 3, and an additional two tenths of pure grade 4. The proportion of grade 3 and grade 4 lesions which results from the presence of an aneurysm may be similarly recorded in the appropriate space on the original data sheet.

That portion of the disease which remains unclassified constitutes grades 1 and 2. Estimation of the part due to subendothelial accumulation of lipid (grade 1) allows grade 2 to be determined by difference. It is in distinguishing lesions of grades 1 and 2 that difficulty is encountered and the choice is sometimes arbitrary. The choice depends upon recognizing whether or not there is a fibrous tissue component diluting the yellow (intensely sudanophilic) color of the plaque and causing a localized elevation. The purely lipidic spots found in the ascending aorta are distinctly elevated, so that feature alone is not a reliable criterion; nor is the superficial localization of lipid a reliable criterion, since deposits may be superimposed upon dense sclerotic plaques and indeed very frequently encircle and outline them.

For those situations where clear distinction between lesions of grade 1 and 2 is not possible, we have adopted the practical expedient of categorizing them as half-grade 1 and half-grade 2. The difficulty is not a major one, however, either from the number of instances where it arises or the maximum error it could create in the atherosclerotic index (an increment not more than nine). Accordingly, the shortcoming should not interfere with the accumulation and comparison of data by different groups. This same conclusion pertains to one other difficulty we have encountered infrequently in the older age groups. This is a more or less diffuse fibrous intimal thickening[6] which is interpreted as atherosclerosis (grade 2) only when there is irregularity and slight nodularity of the surface.

It is also pertinent to mention an intimal structural alteration characterized by slight fibrous thickening in the form of multiple, closely spaced, transverse "striae." These striae are found most commonly in the thoracic aorta below the level of the ligamentum arteriosum, and in the abdominal aorta above the bifurcation and proximal to the ostia of the celiac axis and mesenteric arteries. Deposition of lipid upon these lesions is haphazard and coincidental. Infrequently, they may be greatly hypertrophied and thickened so that they resemble keloids. Having been observed only in infants and children, they presumably become effaced as the intima thickens with maturation and aging; they are not considered part of the atherosclerotic process.

Examination of the coronary arteries begins at and includes the

ostia and extends distally along the epicardial portions of the three major channels as far as it is possible to trace them. Narrowing beyond the ostia can be detected and measured by sequential transection at intervals of 0.5 cm. To establish the atherosclerotic profile, however, it is essential to open the previously transected vessels longitudinally; for this purpose we have used an iridectomy scissors with blunt points.

The degree and extent to which atherosclerosis remains undetected by multiple transection is often surprisingly great. At times narrowing in successive cross sections is due to the same plaque; nonetheless, this situation is recorded as two (or more) points of narrowing. In a schematic inflexible system, a second site of narrowing would not enhance the impairment of circulation more than a single one of equal degree; but *in vivo,* this circumstance would distinctly handicap the possibility of compensatory collateral flow. Calcification may interfere to a variable extent with examination, but it has rarely been necessary to resort to preliminary decalcification. There is obviously no conflict between this type of evaluation and the necessity for removing short segments of vessel for histologic examination.

Cerebral or other visceral arteries may be examined in like fashion. In the brain the vessels examined, all superficial, include the circle of Willis, the stumps of the internal carotid arteries, the vertebral and basilar arteries, and the proximal portions of the anterior, middle, and posterior cerebral arteries. These vessels are most conveniently examined, *in situ,* at the base of the brain after formalin fixation. If the brain is to be examined in the unfixed state, removing, spreading, and orienting the circle of Willis and its tributary branches on stiff blotting paper are essential to maintain relations during preliminary fixation.

Summary

A description has been given of a method for quantitating the atherosclerosis observed at necropsy. Its widespread application and use will allow better comparisons of the extent and severity of the disease in various ethnic, geographic, and economic groups.

Appendix

Charts 1 and 2 are copies of the data and work sheets we have been using. Undoubtedly, other associations and correlations will suggest themselves to others who may then modify them to suit their own purposes. For those pathologists whose obligations and circumstances preclude such elaboration, but who are still interested in the investigation of this problem, we suggest that application of this technique and incorporation of the essential information in their necropsy protocols will provide a more informative record of their observations.

Chart 1

DATA SHEET FOR ATHEROSCLEROSIS STUDY

Acc. no.:

Name: Age: Sex: M.... F.... Abode: Rural.... Urban....

Occupation:

Hospital and location:

Race: White..... Negro..... Indian..... Yellow..... Others.....

Acute accidental death (less than 24 hours):

Physical activity: Hard..... Moderate..... Sedentary.....

MAJOR PATHOLOGIC DIAGNOSIS:

Wasting disease present more than 2 months....., more than 4 mos......, more than 8 mos......

Nutritional status: Severely obese....., Mild to moderately obese.....,
Average....., Thin....., Malnourished.....

Syphilitic aortitis....., Aneurysm (Arteriosclerotic.....) (Syphilitic.....) (Dissecting.....) Rheum. H. D...... Rheum. Arth......

Aortic mural thrombosis....., Cerebral thrombosis....., Mesenteric, renal, or iliac arterial thrombosis.....

Angina pectoris....., Myocardial infarct....., Coronary thrombosis.....

Disabling peripheral arteriosclerosis.....

Hypertension (Established.....) (Presumptive.....) Asthma.....

Diabetes mellitus...., 0 to 5 years...., 6 to 10 years...., more than 11 years....

Alcoholism....., 0 to 5 years....., 6 to 10 years....., more than 11 years.....

Laennec cirrhosis....., Posthepatitic cirrhosis....., Biliary cirrhosis.....

Nephrotic syndrome.....

Hyperthyroidism....., Hypothyroid state....., Hyperlipemia.....,

Hypercholesterolemia.....

Indicate presence of condition or state by a plus mark (+) or its absence by zero (0)

If unknown, insert a minus sign (–)

Chart 2

Acc. no.:

ATHEROSCLEROTIC PROFILE AND INDEX

Name:

Hospital:

	Grade°° 1	Grade°° 2	Grade°° 3	Grade°° 4	
Aorta O, A, B, C, or D°	....× 1/10 +	....+	....× 10 +	....× 10	× 1/30 =
Coronary Arteries O, A, B, C, or D°	....× 1/10 +	....+	....× 10 +	....× 10	× 1/30 =
Cerebral Arteries O, A, B, C, or D°	....× 1/10 +	....+	....× 10 +	....× 10	× 1/30 =

° Circle appropriate one. To calculate index, substitute numerical values 1, 4, 9, 16, or 30 for O, A, B, C, or D, respectively.

Area or disease: Group O — 1/20 or less
Group A — 1/6 or less
Group B — ⅓ or less
Group C — ½ or less
Group D — More than ½

°° The sum of the grades, representing the total disease, always has a value of 10.

Indicate by numbers which add to ten, the proportion of each grade of lesion as follows:

Grade 1 — lipid streaks
Grade 2 — fibrous and lipid plaques
Grade 3 — necrosis, ulceration, and hemorrhage
Grade 4 — calcified plaques

NARROWING INDICES

Select five narrowest foci in cerebral or coronary arteries, gauge degree, check the appropriate column, and calculate as indicated.

DEGREE OF NARROWING°°°

Coronary Arteries	....× 0 +	....× 2 +	....× 8 +	....× 60	× 1/3 =
Cerebral Arteries	....× 0 +	....× 2 +	....× 8 +	....× 60	× 1/3 =

°°° 0 — Residual lumen more than 9/10 of normal
1 — Residual lumen more than ¾ of normal
2 — Residual lumen more than ½ of normal
3 — Less than ½ of the normal lumen remains

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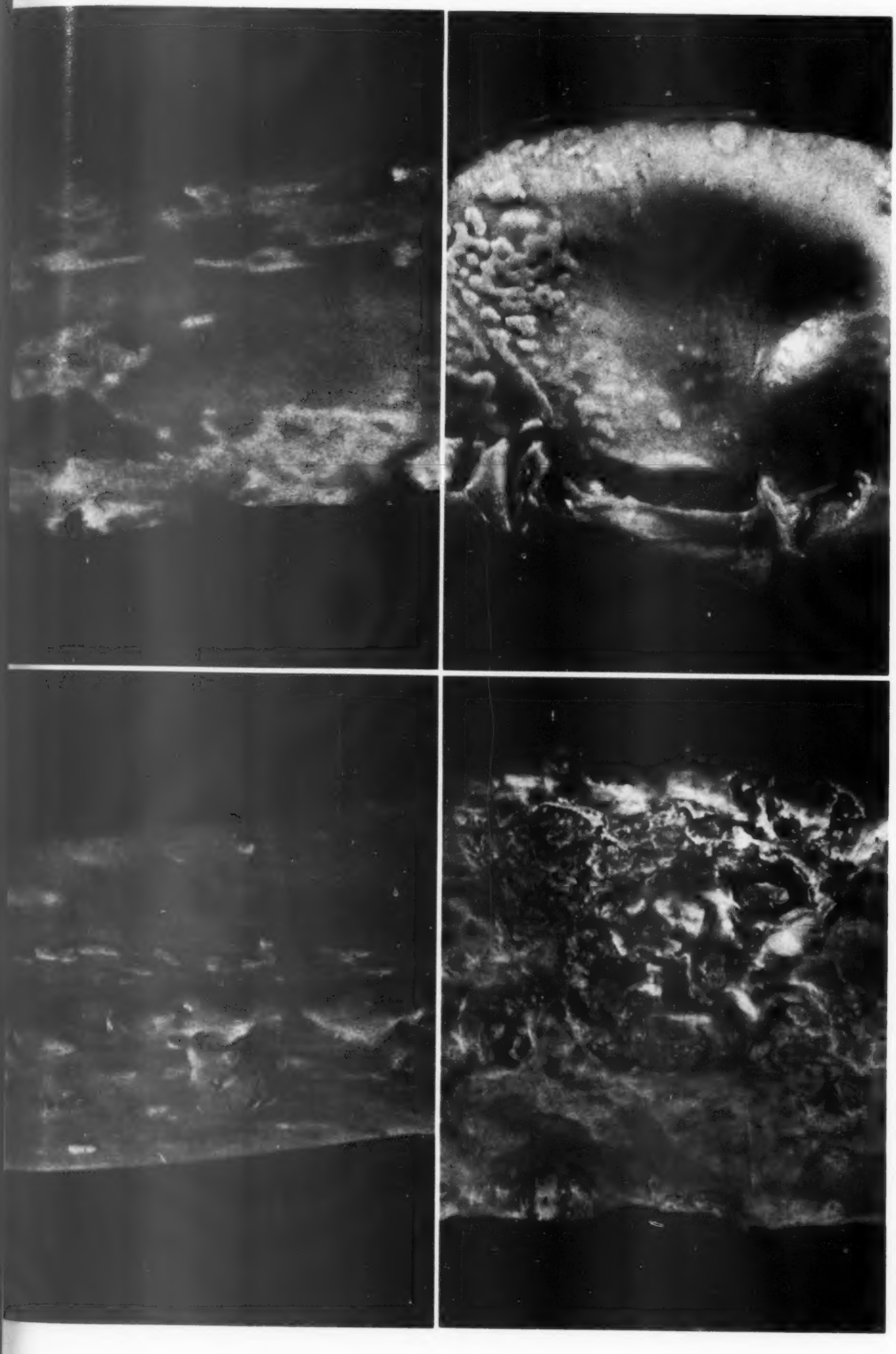
LEGENDS FOR FIGURES

FIG. 1. Internal surface of aorta presenting extensive superficial deposits of lipid in the form of streaks and patches, grade 1 lesions. × 1.

FIG. 2. Intimal surface of supravalvular portion of aorta, showing the sharply delineated, slightly elevated, focal and superficial deposits of pale-staining lipid, grade 1 lesions. Two grade 2 lesions may be observed also, one bordering the coronary ostium showing above the middle valve cusp and the second, centrally, at the distal margin of the aortic segment. × 1.5.

FIG. 3. Intimal surface of aorta presenting several smoothly surfaced, elevated plaques. These grade 2 lesions, most conspicuous about the segmental ostia, are pearly white and contrast with the smaller, more superficial, sharply marginated yellow deposits, grade 1 lesions, in the same field. × 1.

FIG. 4. Intimal surface of aorta with severe atherosclerosis. Only small segments of uninvolved intima are present; the major portion is roughened and thickened by confluent fibrous plaques. The extensive ulceration which characterizes grade 3 lesions may be observed, and the pale superficial lipidic deposits superimposed upon the fibrous plaques. Calcified plaques, grade 4, also present, are not demonstrable photographically. × 1.



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COMPARISON OF ATHEROSCLEROSIS IN GUATEMALA CITY AND NEW ORLEANS *

Carlos Tejada, M.D., and Ira Gore, M.D.

From the Institute of Nutrition of Central America and Panama (INCAP), Guatemala City, Guatemala; the Department of Nutrition, Harvard School of Public Health, and the Department of Pathology, Harvard Medical School, Boston, Mass.; and the Laboratory Service, Veterans Administration Hospital, West Roxbury, Mass.

Clinical experience with the low income group which constitutes the major part of the population of Guatemala has suggested a lower prevalence of atherosclerotic disease than is observed in the United States of America or among Guatemalans of higher income status. Guatemalans with low income tend to be physically active and underweight; they consume a diet which is low in fat and animal protein and high in vegetable protein. They fail to show the gain in weight with age which is common in North America. Racially, they represent Mayan Indian stock variably intermixed with European strains. Their average blood cholesterol is substantially lower than in the population of the United States[1]; values for lipoproteins of the S_f 12 to 20 class and higher, as measured in the ultracentrifuge,[2] fail to show comparable differences.[1]

Because of the current intense interest in serum cholesterol and lipoproteins as possible indices of atherogenic potential, a pathologic study of the actual prevalence of atherosclerosis in the two population groups would help to determine the relative value of the two measurements. Such a study would also help in the evaluation of the influence of other factors, such as the amount and kind of dietetic fat, upon the levels of blood cholesterol and the incidence of atherosclerosis. Present epidemiologic studies of the disease,[3] although they have made noteworthy contributions, admittedly suffer from the lack of a uniform and comparable method of quantitating atherosclerosis. For example, although a low incidence of atherosclerosis has been reported in Japan,[4] Costa Rica,[5] Okinawa,[6] and in the Bantu of South Africa,[7] there is no satisfactory way of comparing these groups with one another. The appraisal procedure outlined previously[8] is believed to have overcome this difficulty and was applied to this study.

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Materials and Methods

The pathologic material available for review consisted of the Sudan-stained aortas from 616 unselected necropsies performed at the Charity Hospital, New Orleans, and 324 performed at the General Hospital in Guatemala. These were examined without knowledge of age, sex, race, or cause of death and classified according to the extent of atherosclerosis and the types of lesions.[8] In this procedure the relative proportion of the total intimal surface involved is estimated and recorded in one of five groups as follows: group O with less than 5 per cent involvement; group A, 6 to 15 per cent; group B, 16 to 33 per cent; group C, 34 to 50 per cent; and group D, 51 to 100 per cent. The proportion of the diseased area represented by each of the four grades of lesion is then determined. These include in grade 1, lipid streaks; grade 2, fibrous atheromatous plaques; grade 3, necrotic, hemorrhagic, and/or ulcerated plaques; and grade 4, calcified lesions. An atherosclerotic index is obtained from these figures by appropriate weighting for both the extent of the disease and the types of lesion which constitute it.[8] The Guatemalan material did not always include the aortic valve, a point to which further reference will be made.

Table I
Aortas Available for Review

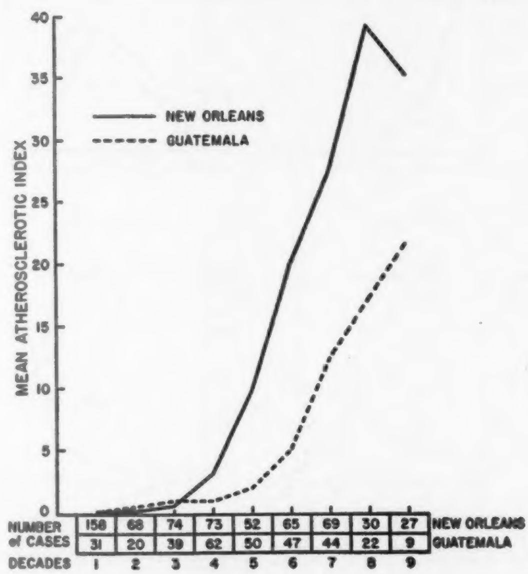
Decade	New Orleans				Guatemala	
	White		Negro			
	Males	Females	Males	Females	Males	Females
1	28	14	67	49	21	10
2	16	9	27	16	16	4
3	20	13	22	19	29	10
4	17	11	21	24	43	19
5	13	11	14	14	34	16
6	20	7	20	18	43	4
7	20	6	22	21	32	12
8	11	5	9	5	17	5
9 or more	7	5	11	4	7	2

Although natural illness was the cause of death in most cases, both groups included a proportion of medicolegal cases with sudden fatalities from traumatic causes in apparently healthy individuals. In Guatemala, all of the necropsy material was obtained from individuals in lower income groups whose dietetic habits are drastically different from those in the United States and from those of upper income mem-

bers of the same country. The sex and age by decade are listed in Table I. Although Strong, McGill, Griffin, and Holman[9] have shown more severe juvenile atherosclerosis in the American Negro, the number of persons examined in the present study did not justify detailed comparison between Negro and white racial groups.

Results

The atherosclerotic index, which has been developed as an expression of the extent and severity of the disease, shows a progressive rise with age in both groups. Under the age of 30 years there are no significant differences between them; the disease appears at an early age in both and is present universally after the second decade (Text-fig. 1). In the first decade, 37 per cent of the aortas of persons from New Orleans

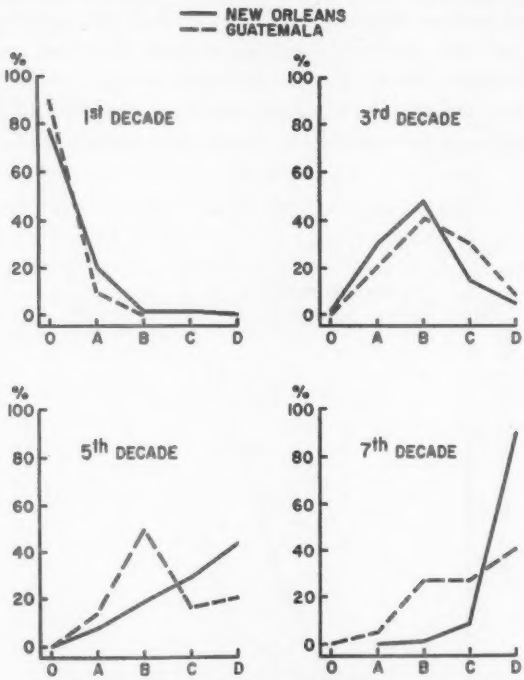


Text-figure 1. Progression of atherosclerosis with age.

and 61 per cent of those from Guatemalans were devoid of atherosclerosis. This discrepancy we believe to be the result of the unavailability of the aortic valve and the supravalvular aortic segment in much of the Guatemalan material. Griffin, Strong, and Holman[10] have pointed out the frequency with which these sites show the initial involvement in juvenile atherosclerosis. In the second decade only 3 and 5 per cent, respectively, were devoid of atherosclerosis. After age 30,

the severity of atherosclerosis increases more rapidly in New Orleans than it does in Guatemala. When the differences between the means were evaluated for each decade above 30 years of age by the t-test, all proved significant at the 1 per cent level, except the last group, 80 years and older, which was significant at the 5 per cent level.

Analysis of the individual factors considered in arriving at the atherosclerotic index serves to specify the nature of the differences



Text-figure 2. Effect of age on surface area involvement.

between the two localities. After age 30 a progressively increasing proportion of individuals present maximal surface involvement (Text-fig. 2). Whereas more than 90 per cent of the New Orleans group above 60 years of age had reached this stage, only about 40 per cent of the same group of Guatemalans were equally affected. Even those older than 80 years had a lesser likelihood of maximal area involvement than the New Orleans group 2 decades younger.

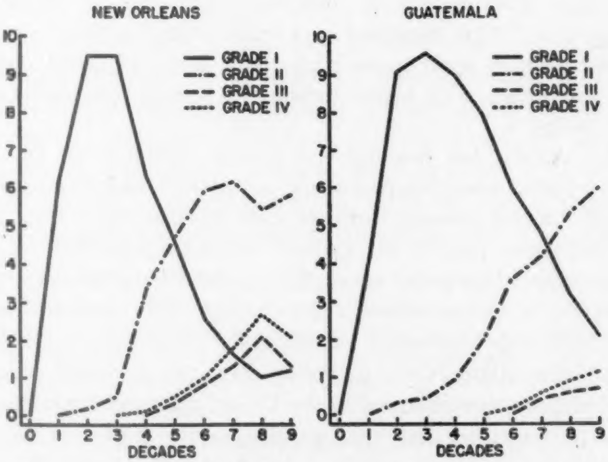
The average relative frequency of each of the four types of aortic lesions by decades in New Orleans and Guatemala is listed in Table II. These percentages do not always total ten to represent ten-tenths, or all, of the disease, as they must in individual cases. One cause for the

discrepancy results from using averages, which, in the first decade especially, include a fair proportion of individuals devoid of atherosclerosis. A second discrepancy occurs in the older age groups in whom an appreciable proportion of the lesions may be both ulcerated and calcified. Although overlapping figures for these two types of lesion

TABLE II

Relative Proportion of Atherosclerotic Lesions (Average Profile)

Decade	New Orleans				Guatemala			
	Grade 1	Grade 2	Grade 3	Grade 4	Grade 1	Grade 2	Grade 3	Grade 4
1	6.2	0	0	0	3.9	0	0	0
2	9.5	0.2	0	0	9.1	0.3	0	0
3	9.5	0.5	0	0	9.6	0.4	0	0
4	6.3	3.3	0	0.1	9.0	0.9	0	0
5	4.6	4.7	0.3	0.5	7.9	2.0	0	0
6	2.6	5.9	0.8	1.0	6.0	3.6	0	0.2
7	1.6	6.2	1.3	1.8	4.8	4.2	0.4	0.6
8	1.0	5.4	2.1	2.7	3.2	5.3	0.6	0.9
9 or more	1.2	5.8	1.3	2.1	2.1	6.0	0.7	1.2



Text-figure 3. Relative frequency of grades of atherosclerotic lesions.

were avoided in calculating the atherosclerotic indices, purposeful duplication, as in Table II, permits comparison of the extent to which calcification occurs.

For both New Orleans and Guatemala (Text-fig. 3), the data show a progressive shift with age from predominance of grade I or lipid

lesions to plaques frequently complicated by necrosis or calcification, or both. However, the change occurs earlier in New Orleans. For example, after age 50, fibrous plaques were predominant in the New Orleans material whereas this was not true in the Guatemalan group until the eighth decade. Ulcerated and calcified plaques appeared in the aortas of the New Orleans group after the third and fourth decades but did not complicate the disease in Guatemala until the fifth and sixth decades. Moreover, the proportion of grade III and IV, ulcerated or calcified lesions increased more rapidly in New Orleans than in Guatemala. In the older age groups in New Orleans, as exemplified by the 70 to 79 age group, almost half the lesions were ulcerated or calcified, or both. By contrast, the process was of similar severity in only 15 per cent of the aortas in Guatemalans of the same age group.

Discussion

The data presented demonstrate a highly significant difference in the severity of atherosclerosis in New Orleans and Guatemala. This becomes obvious and progressively more striking after the third decade. Although the incidence and severity of the disease in both countries are related to age, the fact that there is such a striking discrepancy reaffirms that other factors in addition to age must be considered in atherogenesis.[11] One suggested by a recent study[1] is that Guatemalans with low incomes have appreciably lower levels of blood cholesterol than Guatemalans with higher incomes or sampled populations of the United States.

Unfortunately, too few necropsies were available for the present study to permit adequate pathologic comparison with Guatemalans of the upper income groups. However, each of eight consecutive medicolegal necropsies performed on such subjects presented an atherosclerotic index in the upper range of that exhibited by the corresponding age group of their low-income compatriots.* This limited observation agrees with the impression of clinically significant atherosclerosis among Guatemalans whose activities, diet, and levels of serum cholesterol match those observed in the United States of America.

It is pertinent to note that paralleling the difference in atherosclerosis, over age 40 in the two localities, there were 51 cases of myocardial infarction in New Orleans from a total of 316 cases and only one in the Guatemalan group of 234 cases. On the other hand, of

* These cases, with dietetic habits and activities much like residents of the United States, were excluded from the Guatemalan material being studied.

the eight medicolegal necropsies in Guatemala on individuals of the upper income group, two had myocardial infarcts. Ten instances of aortic mural thrombosis and three arteriosclerotic aneurysms, both complications of severe disease, occurred in the New Orleans material whereas only one of the Guatemalans had mural thrombosis and there were no non-syphilitic aneurysms. The greater prevalence of ulcerated or calcified atherosclerotic lesions, or both, in the United States would appear to explain, at least in part, the difference in incidence of clinically apparent atherosclerosis. An intensive search should obviously be made to identify the environmental factors responsible for the dramatic differences between New Orleans and Guatemala in both atherosclerosis and ischemic heart disease.

Summary

The extent and severity of atherosclerosis in the United States of America and Guatemala were compared by examining the aortas from 616 unselected necropsies in New Orleans and 324 in Guatemala limited to members of the low income group. In both countries the disease started at an early age and was uniformly present after the second decade. Its severity rose progressively with age, but after age 30, the increase was significantly lower in Guatemala. The calcified and ulcerated lesions, common in the older United States group, were also distinctly less prevalent in the material from Guatemala. It is significant that over the age of 40 there were 51 cases of myocardial infarction among 316 cases in the New Orleans material and only one among the 234 Guatemalans. There were also ten cases of aortic mural thrombosis and three of arteriosclerotic aneurysm in the United States material as contrasted to one of mural thrombosis in the Guatemalans.

We are greatly indebted to Drs. Russell Holman, Henry McGill, and Jack Strong of the Department of Pathology, Louisiana State University, for their generosity in making the New Orleans material available for review and extending the facilities of their department to us. The help in collecting aortas by Jorge E. López, Carlos Méndez M., José María Arriola, and Minor Hernández, of the School of Medicine of the University of San Carlos, Guatemala, is also gratefully acknowledged.

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STUDIES ON THE NEPHROTOXICITY OF 5-HYDROXYTRYPTAMINE (ENTERAMINE) IN THE RAT *

LUCIANO FIORE-DONATI and VITTORIO ERSPAMER

From the Institute of Pathological Anatomy, University of Bari, Bari, Italy, and the Institute of Pharmacology, University of Parma, Parma, Italy

Enteramine or 5-hydroxytryptamine (5-HT) produces in rats a marked reduction in flow of urine when administered by intravenous infusion[1] and this effect is more marked after subcutaneous injection.[2-5]

Through the simultaneous evaluation of flow of urine and renal excretion of test substances (thiosulfate, creatinine, para-aminohippuric acid) on large groups of animals, it was demonstrated that antidiuresis following the administration of 5-HT in the rat is due primarily and essentially to a reduction in the glomerular filtration rate. This must be ascribed, in part, to a constriction of the afferent glomerular bed, which leads to a fall in intraglomerular hydrostatic pressure.[2-5]

As early as 1952 and 1953, it was pointed out by Erspamer[6] and Erspamer and Asero[7] that after the administration of high, unphysiologic doses of 5-HT (2 to 10 mg. per kg.) the afferent vasoconstriction produced became visible even macroscopically through the mottled aspect of the surface of the kidney, due to the presence of more or less extensive ischemic areas, interposed between congested areas or areas with an apparently normal blood supply. It was emphasized also that when injury was particularly intense and repeated, ischemia resulted in degeneration and necrosis.

The above observations have been confirmed and extended by Page and Glendening,[8] Hedinger and Langemann,[9] Salgado and Green,[10] and Ballerini and Cantelli.[11]

This paper is designed to give a more nearly complete description of the morphologic changes observed in the kidneys of rats chronically treated with different doses of 5-HT, as well as of rats given a single dose of the substance. Some biochemical and pharmacologic responses presented by these groups of rats have been described and discussed elsewhere.[12]

For a better understanding of the experimental methods and, above all, of the doses of 5-HT used in this investigation, we call attention to some quantitative data. (a) In the rat, the LD_{50} of 5-HT by the intravenous route is 30 mg. per kg.; by the subcutaneous route it is approximately 117 mg. per kg.[13] (b) Following the subcutaneous administration of 25 mg. per kg. of 5-HT, the serum level reaches a

* Received for publication, January 15, 1957.

maximum of 2.8 μg. per ml. within 90 to 120 minutes and remains above normal (0.6 μg. per ml.) for over 48 hours; following the injection of 5 mg. per kg. of 5-HT subcutaneously, the maximum level is 1.26 μg. per ml. and the duration of hyperenteraminemia is approximately 48 hours; finally, following injection of 25 mg. per kg. of 5-HT intraperitoneally, the maximum level is reached within 30 to 60 minutes, but hyperenteraminemia lasts only 10 to 15 hours.[14] (c) The serum of rats treated with daily injections of 5-HT (1 to 15 mg. per kg.) contains throughout the experiment 30 to 250 per cent more 5-HT than the serum of control rats; on the contrary, the 5-HT content of the gastrointestinal mucosa remains unchanged. In rats treated by multiple injections, the antidiuretic effect of 5-HT is reduced both in duration and intensity.[12] (d) The entire rat contains about 400 to 500 μg. of 5-HT per kg. of body weight.[15] This value is considerably higher than that reported previously. The discrepancy is due to the fact that, prior to the studies of Benditt and coworkers[16] and of Parratt and West,[17,18] the very high content of 5-HT in the rat skin was disregarded.

TABLE I

Scheme of Treatment by Multiple Injections

Dose	Grouping of rats	Duration of treatment in days
Daily subcutaneous injections of 1 mg./kg. of 5-HT	Group 1 (5 rats)	30
	Group 2 (5 rats)	68
	Group 3 (5 rats)	91
Daily subcutaneous injections of 5 mg./kg. of 5-HT	Group 4 (4 rats)	23
	Group 5 (4 rats)	30
Daily subcutaneous injections of 10 mg./kg. of 5-HT	Group 6 (3 rats)	3
	Group 7 (5 rats)	12
Daily subcutaneous injections of 15 mg./kg. of 5-HT	Group 8 (5 rats)	25
	Group 9 (6 rats)	40
Daily intraperitoneal injections of 5 mg./kg. of 5-HT	Group 10 (5 rats)	5 to 6
	Group 11 (3 rats)	21
Daily intraperitoneal injections of 10 mg./kg. of 5-HT	Group 12 (5 rats)	4 to 7
	Group 13 (4 rats)	22
Controls	(15 rats)	

MATERIAL AND METHODS

5-Hydroxytryptamine creatinine sulfate* was used throughout the experiments. However, all values are in terms of free base.

Scheme of Treatment by Multiple Injections. Seventy albino rats of both sexes, weighing 120 to 240 gm., were used in the experiments. Unless otherwise stated, the treated animals were sacrificed by decapi-

* Obtained from Farmitalia S.p.A., Milan, Italy.

tation 24 hours after the last injection of 5-HT. The scheme of treatment is summarized in Table I.

Scheme of Treatment by Single Injections. Sixteen rats which received subcutaneously a single dose of 20 mg. per kg. of 5-HT were sacrificed in pairs at intervals ranging from 30 minutes to 24 hours after the injection (group A1); 16 rats which received subcutaneously a single dose of 5 mg. per kg. were sacrificed at intervals, as above (group A2); three other groups of three rats each received, by the subcutaneous route, single doses of 0.5 mg. (group A3), 0.2 mg. (group A4), and 0.1 mg. (group A5) per kg. of 5-HT, respectively, and were sacrificed 1 hour after the injection. Sixteen rats, sacrificed at various intervals after a subcutaneous injection of 0.5 ml. per 100 gm. of distilled water, served as controls.

In order to make visible gross alterations in the intrarenal circulation, a group of 16 rats was given subcutaneously a dose of 10 mg. per kg. of 5-HT. At various intervals after the injections (30, 60, 90 minutes; 2, 3, 5, and 10 hours) the animals were anesthetized with ether, a laparotomy was performed, and the intestines rapidly deflected toward the right. The aorta was exposed and 1 ml. of India ink, diluted one half in saline solution, was rapidly injected counter to the blood current, through a fine needle inserted into the artery just below the origin of the renal arteries. Immediately after the injection the aorta was clamped above the renal arteries and the kidneys were removed for gross and microscopic examination. Six untreated rats, injected with India ink, served as controls.

Finally, a goodly number of rats was injected intraperitoneally and/or subcutaneously with 10 and 100 mg. per kg. of 5-hydroxytryptophan, which is the immediate precursor of 5-HT and can be easily transformed into the corresponding amine by the action of a widely distributed enzyme, the 5-hydroxytryptophan decarboxylase.[19,20]

Histologic Methods. All material for histologic examination was fixed in 10 per cent formol-saline solution. Frozen sections (50 to 100 μ) were stained with benzidine for the differential staining of red blood cells or with Sudan III for fat; sections of the material embedded in paraffin were stained with hematoxylin and eosin, and van Gieson's stain.

The adopted nomenclature for the renal zones was that of Peter[21] and McFarlane.[22]

Results

Rats Treated with Multiple Injections of 5-HT

Macroscopically, animals treated with high doses of 5-HT generally showed kidneys with smooth surfaces and varying degrees of enlargement, often uniformly pale but sometimes irregularly mottled with

TABLE II

Pathologic Findings in Rats Treated with Multiple Injections of 5-HT

	Renal lesions				
	Necrosis	Tubular dilatation	Reparative fibrosis	Casts	Interstitial infiltration
Group 1 (1 mg./kg.×30)					
Rat 1	(+)	+	0	0	0
Rat 2	0	0	0	0	0
Rat 3	0	0	0	0	0
Rat 4	0	0	0	0	0
Rat 5	0	0	0	0	0
Group 2 (1 mg./kg.×68)					
Rat 1	0	++++	(+)	+	+
Rat 2	0	+++	(+)	++	+
Rat 3	0	++++	0	+	+
Rat 4	(+)	+	0	0	0
Rat 5	0	+	0	0	0
Group 3 (1 mg./kg.×91)					
Rat 1	0	++	0	0	0
Rat 2	0	+	0	0	0
Rat 3	0	0	0	0	0
Rat 4	0	0	0	0	0
Rat 5	0	0	0	0	0
Group 4 (5 mg./kg.×23)					
Rat 1	(+)	+	0	0	0
Rat 2	(+)	+	0	0	0
Rat 3	(+)	+	0	0	0
Rat 4	+++	0	+	++	0
Group 5 (5 mg./kg.×30)					
Rat 1	0	+++	0	0	++
Rat 2	0	+	0	0	+
Rat 3	+	+	0	0	++
Rat 4	0	+	0	0	+
Group 6 (10 mg./kg.×3)					
Rat 1	++++	+	0	0	0
Rat 2	+	+++	++	0	0
Rat 3	++	+	0	0	0
Group 7 (10 mg./kg.×12)					
Rat 1	(+)	++++	0	0	0
Rat 2	(+)	++++	++	0	0
Group 8 (15 mg./kg.×25)					
Rat 1	0	++++	0	0	+
Rat 2	(+)	+++	0	0	+
Rat 3	0	+++	0	(+)	(+)
Rat 4	0	++++	(+)	0	+
Rat 5	+	+++	0	0	+
Group 9 (15 mg./kg.×40)					
Rat 1	++	(+)	0	0	0
Rat 2	++	++	0	(+)	0
Rat 3	++	+	0	0	0
Rat 4	++	++	0	0	0
Rat 5	+	++	0	0	0
Rat 6	0	+++	0	0	0
Group 10 (5 mg./kg.×5–6)					
Rat 1	+	+	0	0	+
Rat 2	++	++	+	+	+
Rat 3	++	+++	0	+	+
Rat 4	+	++++	0	+	+

	Renal lesions				
	Necrosis	Tubular dilatation	Reparative fibrosis	Casts	Interstitial infiltration
Group 11 (5 mg./kg.×21)					
Rat 1	(+)	++	+	o	+
Rat 2	o	+	o	o	+
Rat 3	(+)	(+)	o	o	o
Group 12 (10 mg./kg.×4-7)					
Rat 1	+	+++	o	o	+
Rat 2	++	+	o	+	+
Rat 3	++	++	o	+	+
Rat 4	++	++++	+	+	+
Rat 5	+	+++	o	+	+
Group 13 (10 mg./kg.×21)					
Rat 1	+	++	(+)	o	+
Rat 2	+	++	o	+	+
Rat 3	o	+	o	o	o
Rat 4	o	(+)	o	o	+

Number of plus signs indicates severity of lesions; O indicates no lesion; (+) indicates very slight lesion.

pale areas of varying extent. Such areas frequently were bordered by thin, congested, serpiginous lines and, on cut section, they were wedge-shaped and mainly confined to the cortex (Fig. 1); the outermost medulla was affected rarely. Small pits were observed in some cases but usually the kidneys did not appear to be scarred. Benzidine stains showed considerable shortage of blood supply in most cases; sometimes this was uniform and sometimes patchy.

The kidneys of rats treated subcutaneously with 1 mg. per kg. of 5-HT showed no gross changes, except in two animals of group 2. It could always be observed that the difference between the two kidneys of the same animal was merely quantitative.

The histologic pattern in the various groups of rats was so similar as to render it unnecessary to describe each group separately. We wish, however, to stress that remarkable differences in the type and degree of the renal lesions were found not only in different groups but among the individual rats of the same group. Detailed results are summarized in Table II.

The main microscopic lesions were as follows: (a) degenerative changes, generally necrotic in type, affecting the tubular epithelium; (b) tubular dilatation; (c) reparative fibrosis. More rarely, casts of various kinds and calcific deposits, as well as interstitial proliferation and inflammation, also were observed.

The distribution of lesions was consistently focal. It was possible to find areas of relatively normal appearance interspread among the affected regions of even the most severe cases; normal areas were more

frequent at the two renal poles. The renal tissue beneath the fibrous capsule was similarly unaffected even in cases with severe cortical lesions.

Degenerative Changes. As a rule the degenerative changes were confined to the cortex (zone 1) and involved areas varying in size from small foci affecting only a few tubules (Fig. 2) to more extensive involvement (Fig. 3) macroscopically visible as pale areas. These infarct-like areas were sharply demarcated from adjacent normal tissue by narrow strips of hyperemia and hemorrhagic infiltration; they rarely penetrated into the outer strip of the outer zone of the medulla (zone 2).

In most cases, degenerative changes consisted of coagulative necrosis of the proximal convoluted tubules, with loss of nuclei and transformation of cells into acidophilic granular material which completely filled the tubular lumina. The vascular and supporting structures were comparatively free from lesions, especially in the smallest foci. In particular, the glomeruli were only slightly reduced in size and often almost normal. Even the interstitial cells showed no changes other than some hyperchromatism and thinning of the nuclei, probably due to the pressure of enlarged necrotic tubules. There were no substantial changes in the vascular walls, arterial or venous, except where necrosis involved all tissues in the central zones of the largest necrotic areas. No evidence of vascular occlusion was found. In other tubular segments the degenerative changes were less severe, varying from mild cloudy swelling to vacuolation or fatty degeneration.

Comparative study of the various groups of animals demonstrated the renal tubular lesions, and particularly coagulative necrosis, to be more marked, as a rule, after brief treatment than following a long series of injections. Necrosis, however, persisted as disseminated small foci in group 9 after 40 days of treatment.

Tubular Dilatation. Tubular dilatation, one of the most striking and common manifestations of the nephrotoxic action of 5-HT, appeared, in contrast to necrosis, to be often more prominent in rats treated over long periods (Fig. 4). It was focally distributed, as were the necrotic lesions, and usually involved large areas of renal parenchyma. When dilatation and necrosis were both present in the same kidney, they were found to be adjacent and to affect different groups of nephrons. Zone 2, which consists mainly of the straight parts of the proximal convoluted tubules, was the most common site of dilatation (Fig. 5), and in this zone changes could be observed in the very early stages. In many cases adjacent zones (1 and 3) also were affected.

Very often the dilated tubules assumed a cystic form, which probably resulted from the rupture and disappearance of the dividing wall between two or more neighboring tubular cavities. The remains of the broken walls were visible as thin, incomplete septa (Fig. 6).

The lumina of dilated tubules were frequently empty. No evidence could be found of distal tubular compression or obstruction capable of giving a mechanical explanation of the dilatation of proximal tubules. Calcareous material filling the lumina of distal segments was detected in only one case (group 9). Where tubular dilatation was more marked, a certain amount of dilatation was observed also in the glomerular space, especially in the juxtamedullary glomeruli (Fig. 5).

Dilated tubules were lined with either cuboidal or, more commonly, flattened epithelium; the more dilatation, the flatter the epithelium. No regenerative changes, such as those reported by other investigators,[23] could be observed in our cases. Sometimes atrophic tubules were interspersed among those showing dilatation.

Reparative Fibrosis. When fibrosis was observed, it appeared as circumscribed wedge-shaped areas confined to the cortex and corresponding in site, shape, and size to the foci of necrosis. Fibrosis was particularly evident in cases showing contemporaneous necrotic changes, and there is little doubt that it should be considered as a healing of the necrotic process (Fig. 7). The fibrotic areas were made up of young connective tissue, some atrophic tubules, vessels with thickened and partially hyalinized walls, and often glomeruli. These appeared in most cases well preserved or showed inconspicuous morphologic changes, but were closely packed as a result of the shrinkage of the fibrotic tissue.

Other Signs of Renal Injury. In some cases, in addition to the main changes mentioned above, granular or colloid-like casts were found. They never filled the lumina of tubular segments dilated or otherwise included in areas showing dilatation. In other cases, interstitial inflammation was noted, and was sometimes well marked. Usually situated in the cortex or in zone 2, it consisted of lymphocytes, plasma cells, and histiocytes.

Three rats of group 7 were sacrificed 20 days after the treatment was discontinued, in order to permit study of the further evolution of the lesions in the absence of exogenous 5-HT. In contrast to those treated with the same amount of 5-HT but sacrificed immediately after treatment, these rats showed no distinctive renal changes other than slight dilatation of the tubules of zone 3 and rare areas of reparative fibrosis.

In summary, prolonged subcutaneous treatment with varying amounts of 5-HT, over periods ranging from 4 to 91 days, provoked bilateral renal lesions in most animals. Generally, the severity of the renal injury was less conspicuous at a lower dosage, but in three rats of group 2 (1 mg. per kg. over 68 days) the histologic pattern could be compared in every respect to that observed in animals treated with 10 or 15 mg. per kg. of 5-HT.

Other organs examined (e.g., heart, liver, spleen, lungs, stomach, and intestines) showed no abnormality, thus indicating the selective vulnerability of the kidney to the amine.

Rats given intraperitoneal injections (groups 10, 11, 12, and 13) exhibited almost the same renal lesions as those under subcutaneous treatment. The only difference noted was a greater severity of the renal injury, which in some cases involved almost the whole kidney. In one animal, necrosis reached deeply into the inner zone of the medulla.

In a few cases anemic infarcts were observed in the spleen, and foci of hemorrhage, edema, and necrosis were seen in circumscribed areas of the mucosa and submucosa of the stomach.

Rats Treated with Single Injections of 5-HT

Group A1 (20 mg. per kg.). In comparison with those of the control animals (Fig. 8), the kidneys of all rats sacrificed 1 hour after the administration of 5-HT showed very conspicuous and diffuse congestion. This was especially striking in the cortex (Fig. 9), where glomeruli, intertubular capillary network, and interlobular and arciform veins were dilated and engorged with red blood cells. Congestion was also very marked in zone 3, where the arteriolae and venulae rectae are situated. Congestion in zones 2 and 4, which normally have less blood supply than the other zones, was less remarkable, though always greater than in the control rats.

After 3 and 5 hours, the circulatory pattern was similar to that just described, but congestion was less prominent. Some differences could be noted between the cortical and the juxtamedullary glomeruli, the former being more congested. After 10 hours the kidney was almost normal, with the exception of some circumscribed areas of ischemia in the cortex and some persistent congestion at the level of the cortical glomeruli.

Group A2 (5 mg. per kg.). Circulatory changes in group A2 were essentially similar to those observed in group A1, but more moderate. A diffuse congestion appeared within 30 minutes, seemed increased after 2 hours, then diminished gradually, until it disappeared after 10

to 24 hours. In this group, too, the kidneys of rats sacrificed after 5 and 10 hours exhibited ill-defined areas of relative ischemia affecting the cortex.

No appreciable circulatory changes could be observed in rats injected with doses of 5-HT less than 1 mg. per kg. (groups A3, A4, and A5).

Certain histologic findings noted in groups A1 and A2 are not easy to account for, and deserve further attention. Early degenerative changes were observed in the tubules of zone 2, especially in the straight limbs, of rats sacrificed after 1 and 2 hours. The epithelial cells were swollen and showed granular cytoplasm, which occasionally was partially transformed into acidophilic material discernible in the tubular lumina. Veins of medium and small size (especially interlobular and arciform) and capillaries were conspicuously enlarged and grossly engorged by red blood cells of conglutinated appearance (Fig. 10). The arciform and interlobular arteries, as well as the afferent arterioles showed slight narrowing of the lumina and mild thickening of the walls, but exhibited no morphologic changes. Such features are probably the result of a vascular spasm. It was especially noteworthy that plasmatic transudation was discernible around the capillaries (Fig. 11) and less frequently around the veins of rats sacrificed after 2 hours. Five hours after injection, congestion was reduced but tubular dilatation in zone 2 became more marked. Every case showed such dilatation but the degree varied even from area to area in the same kidney. The glomeruli appeared slightly enlarged with the capillary loops somewhat dilated.

After 8 hours, histologic changes, with the exception of glomerular enlargement, were less prominent. All glomeruli exhibited enormous dilatation of the tufts and complete obliteration of the capsular spaces (Fig. 12). After 10 and 24 hours, the histologic pattern was indistinguishable from that of the control animals except that in the rats sacrificed after 10 hours a mild enlargement of the glomeruli still persisted.

Circulatory and structural changes noted in groups A1 and A2 are summarized in Table III.

Experiments with Injections of India Ink

The intrarenal circulatory changes observed in groups A1 and A2 were confirmed by the *in vivo* renal perfusion with India ink.

Soon after the kidneys of control rats were injected with India ink, they became uniformly blackened; on section the cortex and medulla

were gray-black. Microscopically, ink was seen within every glomerulus and was distributed uniformly throughout the peritubular capillaries and other vessels.

The kidneys of the rats sacrificed 30 to 45 minutes after the injection of 10 mg. per kg. of 5-HT appeared to be mottled gray-black with pale cortical areas of various extension visible on the surface and on cut section. One hour after the injection most of the cortex was pale; only rare radial segments and sometimes renal poles appeared to be perfused with ink. Microscopically, no India ink could be seen in most of the cortical glomeruli and the capillary peritubular network; some juxtamedullary glomeruli, which appeared engorged with blood, and

TABLE III

Acute Renal Changes Observed Following Single Subcutaneous Injections of 5 and 20 mg. per kg. of 5-HT

Time after injection	Renal changes			
	Congestion	Tubular degeneration	Tubular dilatation	Glomerular enlargement
30 minutes	++	o	o	o
60 minutes	++++	o	o	o
2 hours	+++	+	o	o
3 hours	++	+	(+)	o
5 hours	+	(+)	+	+
8 hours	(+)	o	o	++++
10 hours	(+)	o	o	++
24 hours	o	o	o	o

O indicates no change; (+) indicates very slight change; + indicates evident changes, of different severity.

the vasa recta contained small amounts of ink mixed with blood. Two hours after the injection of 5-HT small, pale areas persisted in the cortex; after 3 hours the observations were almost the same as in control animals.

Rats Treated with 5-Hydroxytryptophan

Twenty rats were injected with 100 mg. per kg. of DL-5-hydroxytryptophan (50 mg. per kg. intraperitoneally and 50 mg. per kg. subcutaneously), and at the same time a dose of 5 ml. of tepid tap water, per 100 gm. of body weight, was given by stomach tube in order to establish the effect of the drug on water diuresis.

Fifty per cent of administered water was eliminated only within 5 hours, instead of within 90 minutes, as in control rats. The animals were sacrificed after 24 hours. In all cases the kidneys showed signs

of severe injury. Macroscopically, the organs appeared enlarged with the outer and cut surfaces clearly mottled; microscopically, tubular degeneration was prominent. The 24-hour specimen of urine contained very large amounts of 5-hydroxyindoleacetic acid, the main metabolite of 5-HT.

In three rats which received a dosage of 10 mg. per kg. of 5-hydroxytryptophan subcutaneously, antidiuresis was inconstant and less intense. Upon gross and microscopic examination, the kidneys showed no alteration, with the exception of lightly mottled surfaces.

These preliminary experiments are of interest in so far as they demonstrate that the renal lesions obtainable with 5-HT may be produced also by its immediate precursor 5-hydroxytryptophan, which is decarboxylated in the organism to the active, nephrotoxic amine.

Discussion

Present results, together with those obtained by other investigators, afford definite evidence that parenteral administration of 5-HT or of the 5-HT precursor, 5-hydroxytryptophan, provokes foci of cortical necrosis in the rat kidney, which may later undergo fibrotic change. Tubular dilatation observed with the necrotic lesions is more marked in animals given the most prolonged treatment. No thrombotic lesions or primary vascular changes were observed. On this last point, however, our observations are at variance with those of Page and Glendening.[8] This may depend in part on the different route of administration of 5-HT, since the above investigators gave the amine by continuous intravenous infusion (1.7 mg. of 5-HT per kg. and per hour for 5 to 7 hours).

Necrosis principally affected the proximal convoluted tubules and was coagulative in type. The lesions penetrated deeply into zone 2 in only a few cases. The severity of the renal damage and the prevailing kind of lesion varied considerably from one animal to another of the same group.

It is important to emphasize that there was no direct relation between intensity and duration of treatment and the severity of the renal lesions. In some cases, indeed, after brief but sufficiently intensive treatment, more severe lesions were observed than could be noted after prolonged treatment. It is very probable that the most serious renal damage is caused by the early or even the first injections of 5-HT, and that as treatment continues, the kidney becomes less and less sensitive to the toxic agent. This opinion is corroborated by our previous pharmacologic experiments[12] which demonstrated that in rats treated with

multiple injections of 5-HT the strong antidiuretic effect possessed by the substance is reduced both in intensity and duration, or even suppressed. A progressive desensitization of the renal effectors to the 5-HT, which is continuously present in the blood in amounts above normal, was believed to be the cause of the phenomenon.

It is worthy of consideration that remarkably serious and extensive lesions were sometimes produced even by relatively small doses of 5-HT (1 mg. per kg.).

Coagulative tubular necrosis has been induced experimentally in different animal species by a number of investigators by the use of mechanical occlusion of the renal vessels, particularly of the renal artery,[23,24,25,27,28] by pitressin,[31,32] staphylococcal toxin,[33] and traumatic shock.[26,29,30,34] All writers agree in considering this kind of necrosis as a direct consequence of renal ischemia.

Opinions differ, however, as to the interpretation of the pathogenesis of the ischemia. The majority of observers consider the ischemia to be the consequence of an arterial spasm, which affects in particular the interlobular and afferent arteries[32,35]; others assume the intervention of a venous spasm, presumably of the venules, with the consequent stagnation hyperemia and functional ischemia.[33]

Starting from the purely morphologic data outlined above, it is rather difficult to decide whether ischemia and cortical necrosis provoked by 5-HT are attributable to venous or arterial spasm; but for the following reasons we are inclined decidedly to support the second hypothesis. (a) Necrotic changes observed are very similar in type and distribution to those obtained by temporarily cutting off the arterial flow.[25,28] (b) Hemorrhage following the surgical removal of one pole of a rat's kidney is considerably less in rats pre-treated with 5-HT than in control animals.[15] (c) Even extremely small doses of 5-HT (80 to 200 μg. per kg.), which fail to produce morphologic changes, provoke a marked reduction of the glomerular filtration rate, and, contemporaneously, of the renal plasma flow.[2] Both phenomena can easily be explained by a spasm of the afferent glomerular bed, but not so easily by a venous spasm. In this case we should expect to find that the increased hydrostatic pressure prevailing in the capillary network of the glomerulus, as a consequence of the increased downstream resistance, would result in an augmented glomerular filtration or, at least, in an evident dissociation of the behavior of the glomerular filtration rate from that of renal plasma flow. (d) It has been observed in rats that hemorrhage following partial hepatectomy, abdominal cutaneous wounds, or amputation of the tail is considerably less when animals

had been pre-treated with 5-HT.[36-38] (e) Finally, 5-HT injected intra-arterially in the human forearm causes a constriction of the arterioles and a dilatation of capillaries.[39]

All this leads us to maintain that 5-HT acts to provoke a spasm of the afferent glomerular bed, and to interpret the capillary and venous congestion as the consequence of either anoxemic paralysis following arterial spasm, or dilatation of the capillaries and veins directly provoked by 5-HT, or of both factors.

It is difficult to explain the tubular dilatation we have found so often in the kidneys of our rats. Other investigators,[23,25,27,40] who have observed this dilatation in experimental nephropathies comparable to that produced by 5-HT, postulate mechanical obstruction[40] (e.g., casts or calcareous deposits), or else compression[27] (interstitial edema or fibrosis), or, finally, elimination of abundant necrotic epithelia.[23] We are unable to decide which of these theories applies to our material nor do we know whether another theory might best explain the facts. The possibility, for example, can not be ruled out that dilatation should be interpreted as the result of hyperfunctioning of the surviving nephrons. If so, the phenomenon would be comparable to that observed in human sclerosis of the kidney or in bilateral renal hypoplasia.[41]

The focal character of the lesions, radially distributed in the cortex, is not peculiar to 5-HT nephropathy, as it has been found in all other forms of ischemic-necrotizing renal injury, whether experimentally or spontaneously produced. It is highly probable that this particular distribution of the lesions should be attributed essentially to the varying responsiveness of different sectors of the cortical vascular tree to 5-HT.

Experimental pharmacologic data on the elimination of water, creatinine, and para-aminohippuric acid by the animals treated with 5-HT suggest that all, or nearly all of the afferent glomerular vessels undergo a more or less intense constriction (the temporary complete suspension of the diuresis could not otherwise be explained). The anatomico-pathologic findings, on the other hand, prove that intensity and, above all, duration of the vascular spasm must differ from one artery to another.

Byrom's observations[32] on pitressin may perhaps be applied to 5-HT, i.e., that small doses of 5-HT affect only the afferent glomerular arteries, while larger doses affect the larger arteries as well.

Another interesting finding which is not easily interpreted is the relative survival of the glomeruli included in the necrotic areas. Other investigators[26,27,32] have observed a similar phenomenon. The most

plausible explanation seems to be that the glomerular vascular structure is far more resistant to ischemic anoxia than are the delicate tubular epithelia.

The most peripheral subcapsular zone of the cortex is often relatively unaffected. We can certainly attribute this behavior, confirmed by various research workers,[8,9,32] to the existence of an adequate blood supply through the capsular vessels on which 5-HT has evidently no effect.

We have never observed corticomedullary shunts similar to those described by others in experimental and human necrotizing nephropathy (cholera, hemorrhagic fever, renal cortical necrosis).

Previous pharmacologic evidence[2] demonstrated that the intrarenal arterial tree of the rat is particularly sensitive to 5-HT. Indeed, subcutaneous doses as low as 5 to 10 μg. per kg., which were quite ineffective on other vascular areas and 50 to 100 times inferior to the minimal dose active on the systemic blood pressure, were sufficient to produce an evident antidiuresis, caused by afferent vascular spasm. The results of the present investigation once again confirm the highly selective action of 5-HT on the rat kidney. In fact, the only lesions observed in animals treated with 5-HT were renal, except for a few rats given high intraperitoneal doses of the substance, where infarcts of the spleen and necrotic areas in the gastric mucosa were noted.

The conclusions reached in this paper are valid only for the rat and cannot be generalized without adequate experimental evidence. This statement should be maintained in spite of the observation of Page and Glendening[8] that intravenous injections of 5-HT provoke a visible blanching of the kidneys of guinea pigs and rabbits as well, and the observation of Abrahams and Pickford[42] that following 26 μg. per kg. of intravenous 5-HT the dog kidney blanches for several minutes and when bromophenol blue is injected subsequently, it is found abundantly in the medulla but only in a few radial streaks in the cortex.

It is possible that 5-HT formed in the organism by slow decarboxylation of the injected precursor 5-hydroxytryptophan, and preserved from excessively rapid breakdown by means of the amine oxidase inhibitor iproniazid, is capable of injuring the kidneys of other more resistant animal species. Investigation in this field is in progress.

As previously pointed out, renal lesions very similar to those provoked experimentally in the rat by 5-HT have repeatedly been found in human pathology. White[43] reported one of the earliest and most interesting examples, and Page and Glendening,[8] more recently, insisted on the close resemblance between the renal lesions observed in rats fol-

lowing the administration of 5-HT and those found at necropsy in pregnant women who died more than 24 hours after the occurrence of severe abruptio placentae. For the moment, however, there is no convincing evidence that 5-HT plays a rôle in the pathogenetic mechanism of human cortical necrosis, even should 5-HT be liberated in excess or be allowed to act on an abnormally sensitized substratum.

Summary

5-Hydroxytryptamine administered by single or multiple injections to the rat, in subcutaneous or intraperitoneal doses of 1 to 20 mg. per kg., is capable of provoking a more or less severe renal injury. This consists essentially in coagulative necrosis of the proximal convoluted tubules and/or conspicuous tubular dilatation. The lesions, which are mainly focal in distribution, may result in complete repair or in fibrotic scarring, according to their severity. A nephrotoxic action identical with that possessed by 5-HT is shown by the 5-HT precursor, 5-hydroxytryptophan.

Conspicuous differences are found in the degree of renal injury not only between one animal and another, but even between the two kidneys of a single rat. Normally, the higher the dose, the more severe the lesions; the length of treatment, on the contrary, seems to be of minor importance. It is probable that the greatest renal injury is caused by the first injections of 5-HT. The kidney then seems to become progressively less sensitive to the substance and it even seems possible that recovery from the initial injury may already have begun while the daily injections were still being given.

Cortical necrosis from 5-HT is certainly due to protracted ischemic periods. Furthermore, it is probable that ischemia is consequent to arterial spasm, possibly affecting interlobular and afferent glomerular arteries. This opinion is supported by the close similarity which exists between 5-HT lesions and lesions obtainable by temporary artificial obstruction of the renal artery, and also by the results of a number of pharmacologic experiments.

Capillary and venous congestion of the kidney seen after 5-HT is not due to a venous spasm, but must be considered either as a paralytic manifestation consequent to anoxia provoked by the arterial constriction, or as an active phenomenon resulting from a direct dilatatory action of 5-HT on capillaries and veins.

The injurious effect of 5-HT on the rat kidney is highly selective. Apart from exceptional cases, no other tissue or organ is appreciably

affected by the substance. These results are in full accordance with those obtained in pharmacologic experiments.

Owing to the demonstrated existence of striking species differences in the vulnerability of the kidney by 5-HT, the results obtained in the rat cannot be extended to other animals. Similarly, the resemblance of the morphologic findings does not seem a sufficient criterion for considering 5-HT as a factor participating in the pathogenesis of some renal lesions found in human pathology.

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LEGENDS FOR FIGURES

FIG. 1. Cut surface of ischemic kidney with infarct-like area in the cortex, sharply outlined by a narrow zone of hyperemia. Group 6 (10 mg. per kg. of 5-HT subcutaneously). Benzidine stain. × 6.

FIG. 2. Scattered cortical foci of necrosis involving small groups of tubules. Group 6 (3 × 10 mg. per kg. of 5-HT subcutaneously). Hematoxylin and eosin stain. × 110.

FIG. 3. Extensive area of coagulative necrosis in the cortex. Group 6 (3 × 10 mg. per kg. of 5-HT subcutaneously). Hematoxylin and eosin stain. × 110.

FIG. 4. Tubular dilatation especially involving zone 2. Moderate thinning of the cortex. Group 7 (12 × 10 mg. per kg. of 5-HT subcutaneously). Hematoxylin and eosin stain. × 40.

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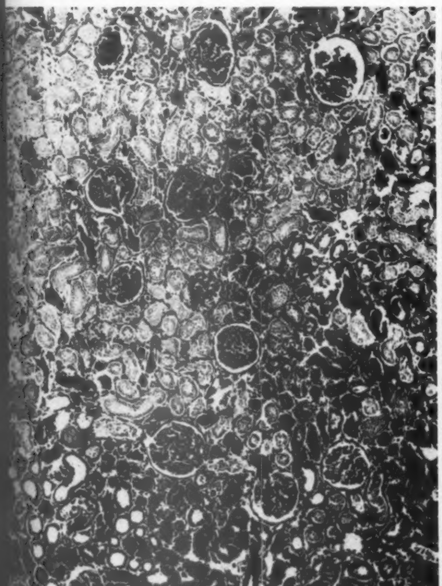

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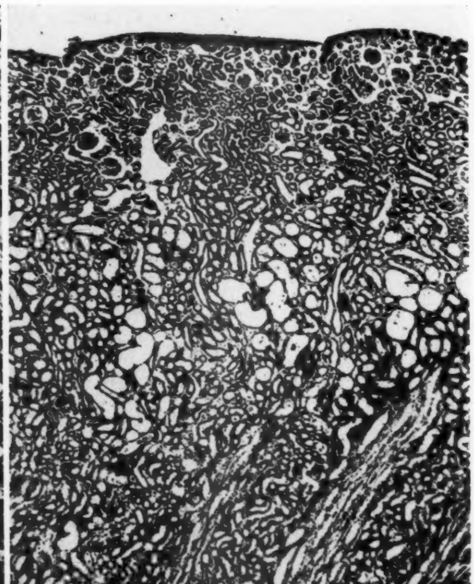

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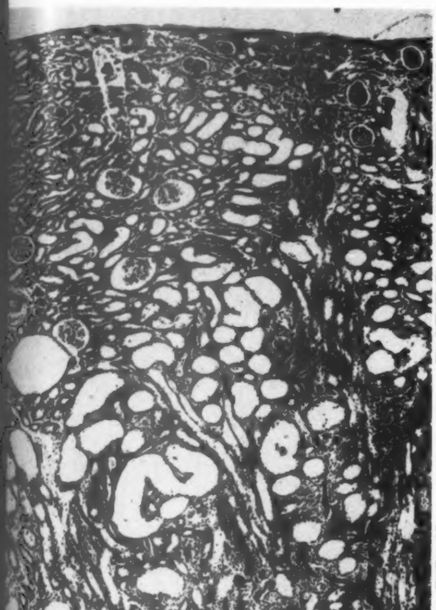
FIG. 5. Well marked dilatation of the tubules and of the capsular space of some glomeruli. Group 6 (3 × 10 mg. per kg. of 5-HT subcutaneously). Hematoxylin and eosin stain. × 40.

FIG. 6. Striking tubular dilatation: the lumen is quite empty and lined by flattened epithelium. Group 5 (30 × 5 mg. per kg. of 5-HT subcutaneously). Hematoxylin and eosin stain. × 380.

FIG. 7. Cortical wedge-shaped area of scarring in immediate contact with a zone of tubular dilatation. Group 7 (12 × 10 mg. per kg. of 5-HT subcutaneously). Hematoxylin and eosin stain. × 40.

FIG. 8. Vascular pattern of cut surface of kidney from a control animal. Benzidine stain. × 6.



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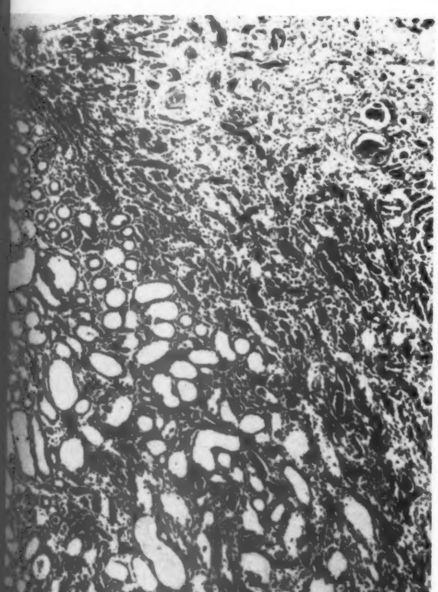

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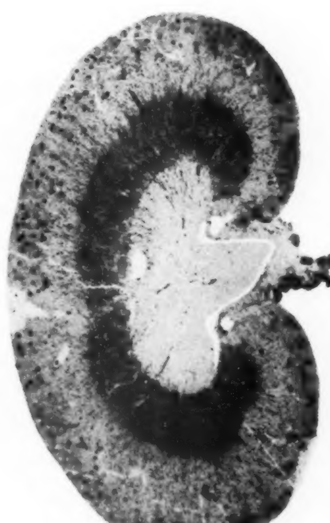

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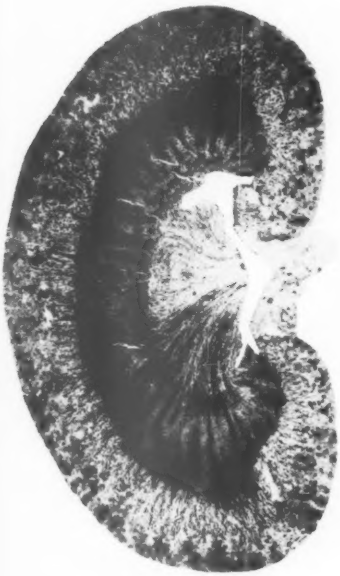
FIG. 9. Marked and fairly uniform congestion of the kidney seen 1 hour after a single injection of 5 mg. per kg. of 5-HT subcutaneously. Benzidine stain. × 6.

FIG. 10. Peritubular capillaries engorged by partly fused erythrocytes, 30 minutes after a single subcutaneous injection of 5 mg. per kg. of 5-HT. Hematoxylin and eosin stain. × 435.

FIG. 11. Transudated plasmatic fluid around capillaries in zone 2, 2 hours after a single injection of 5 mg. per kg. of 5-HT subcutaneously. Hematoxylin and eosin stain. × 420.

FIG. 12. Very marked dilatation of all capillary loops. Enlargement of glomerular tuft and complete obliteration of the capsular space may be noted. Eight hours after a single subcutaneous injection of 5 mg. per kg. of 5-HT. Hematoxylin and eosin stain. × 420.



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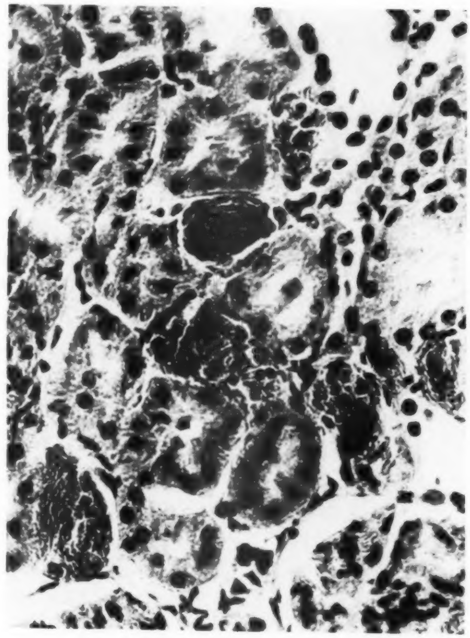

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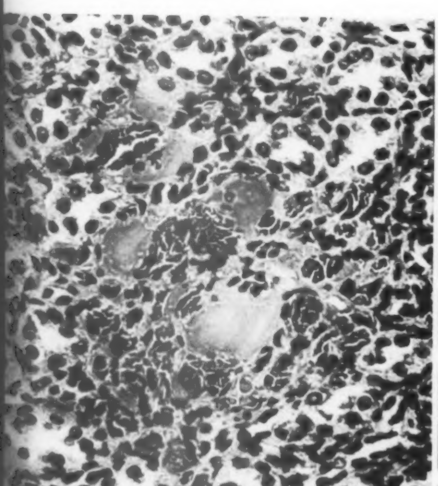

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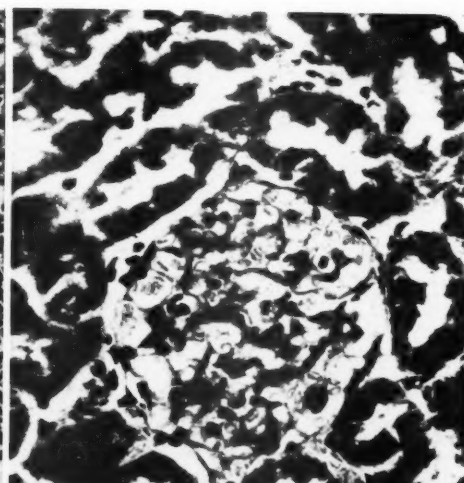

12

EFFECT OF HYPOPHYSECTOMY AND SUBSTITUTION THERAPY WITH STH UPON EXPERIMENTAL BONE LATHYRISM *

Hans Selye, M.D., and Joaquin Ventura, M.D.
From the Institut de Médecine et de Chirurgie expérimentales, Université de Montréal, Montreal, Que.

Aminoacetonitrile (AAN) is an analogue of the $\beta(\gamma$-L-glutamylamino)propionitrile which occurs in the seeds of *Lathyrus odoratus* and which presumably accounts for the toxic effects of this plant upon the skeleton (Dupuy and Lee,[1,2] Schilling and Strong[3]). Both these nitriles, when fed to or injected into rats, produce numerous exostoses, especially at the sites of muscle insertion, and an irregular proliferation of cartilage, particularly in the rapidly growing epiphyseal plates. Although the presence of AAN itself has not been demonstrated in *Lathyrus odoratus* seeds, it is even more effective in inducing skeletal changes than the naturally occurring $\beta(\gamma$-L-glutamylamino)propionitrile (Wawzonek *et al.*[4]).

The literature on bone lathyrism has been the subject of an excellent review (Strong[5]), and need not be discussed here in detail. Suffice it to add that more recently we have learned that the response of bone, either to diets containing *Lathyrus odoratus* seeds or to subcutaneously administered AAN, can be influenced at will. For example, cortisol, ACTH, and thyroxine inhibit, while STH (somatotrophic hormone) or partial hepatectomy augment the characteristic bone lesions produced by lathyrogenic substances (Selye,[6] Selye and Bois[7-10]). Additional experiments on rats revealed that AAN produces a metabolic defect which causes the skeleton to respond with excessive callus formation following local injury (Selye[11]).

In view of these observations, it seemed of interest to determine whether the sensitivity of the bones to AAN is affected only by excessive (pharmacologic doses of STH, or whether even the normal hormonal secretion of the hypophysis raises the susceptibility of bones to lathyrism above that of hypophysectomized animals. To further characterize the rôle of STH, it also appeared essential to verify whether, in the absence of the pituitary gland, substitution with STH alone would suffice to augment AAN sensitivity above the basic level.

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Experimental Methods

Forty female Sprague-Dawley rats, having an initial body weight of 91 to 111 gm., were subdivided into three groups, each with an average body weight of 101 gm. All three groups received the same amount of AAN, but group I served as controls, group II was hypophysectomized, and group III was hypophysectomized and subsequently treated with STH.

Hypophysectomy was performed through the parapharyngeal route, under ether anesthesia, on the first day of the experiment. The animals of all three groups received one subcutaneous injection of 400 μg. of cortisol acetate microcrystals (Cortril, Pfizer) suspended in 0.2 ml. of water. Previous observations had shown that such transitory treatment with a glucocorticoid greatly enhances the resistance of hypophysectomized rats during the postoperative period. Of course, only groups II and III were in need of this treatment, but for the sake of uniformity the non-hypophysectomized rats of group I were given the same amount of cortisol as the others.

AAN was administered in the form of aminoacetonitrile hydrosulfate in aqueous solution, beginning on the third day after hypophysectomy. The initial daily dose was 1 mg., this amount being raised by 1 mg. every day until the sixth day. After that, injections were given twice daily, and the amount was raised by 2 mg. per day until the total dose of 10 mg. was reached. Each dose was injected subcutaneously in 0.2 ml. of water.

STH (Armour Laboratories) was given subcutaneously at the dose of 500 μg. in 0.2 ml. of water twice daily, beginning on the sixth day after hypophysectomy.

The dose of AAN was raised gradually, and STH was started several days after hypophysectomy, because preliminary experiments had taught us that, immediately after ablation of the pituitary gland, rats have a very low resistance to both AAN and STH.

On the eleventh day, the skin around the left knee joint was shaved, and, under ether anesthesia, the tibia was transected, with a pair of Liston bone forceps, about 2 mm. below the cartilage of the epiphyseal junction, care being taken not to injure the fibula which was to act as a splint. This was done in order to explore the effect of hypophysectomy and of STH upon AAN-induced formation of giant callus. Prior to the bone transection, AAN administration was interrupted for 24 hours, because the drug tends to predispose to excessive bleeding, but immediately after the intervention, AAN treatment was again resumed.

Throughout the experiment the animals were fed on Purina Fox Chow and Pablum.

On the 20th day after hypophysectomy, the animals of all groups were sacrificed with chloroform, and both tibias, as well as the right femur, were fixed and simultaneously decalcified in Susa solution for subsequent histologic examination. After being embedded in paraffin, sections 6 μ in thickness were stained with hematoxylin and eosin.

Results

Between the third and fourth day after fracture of the tibia, digital palpation showed that the callus was much more voluminous in the intact and in the hypophysectomized-STH-treated rats than in the merely hypophysectomized animals. This became even more obvious at necropsy. After removal of the skin in the hypophysectomized rats which received no STH treatment, callus formation at the site of the tibial fracture was minimal, although in many animals of this group, hemorrhages occurred at the site of the bone lesion. On the other hand, in the intact and in the hypophysectomized-STH-treated rats, callus formation was excessive, and the newly formed bone appeared to invade the surrounding muscles, more or less diffusely, so that it was difficult to dissect the skeletal structures from the adjacent soft tissues (Fig. 1).

Other parts of the skeleton of the intact and of the hypophysectomized-STH-treated animals also showed the occurrence of exostoses and exuberant periosteal bone formation (especially in the ribs, most of the tubular bones, the atlas, and the occiput), but only very minor lesions of this type could be detected in the hypophysectomized rats receiving no STH. This difference in reactivity was particularly manifest in the mandibles, which, presumably because they are the site of many muscle insertions, have a great predisposition to exhibit signs of bone lathyrism (Fig. 2).

Upon histologic examination, it was found, both at the site of the tibial fracture and elsewhere throughout the skeleton, that the proliferation of connective tissue, and its transformation into cartilage and bone, was much more intense in the intact and in the hypophysectomized-STH-treated, than in the hypophysectomized animals. Interestingly, the junction-cartilage plates, which are normally quite atrophic in hypophysectomized animals, showed some signs of stimulation by AAN, even in the rats of group II. The total width of the cartilaginous plate was increased, owing to an excessive development

of chondrocytes and intercellular substance; furthermore, cleft formation (which usually precedes the slipping of epiphyseal disks in lathyrism) occurred, even in the junctional cartilages of the hypophysectomized rats receiving no substitution therapy, although to a much lesser extent than in the other two groups (Figs. 3 to 5).

Along the shafts of the long bones, periosteal new-bone formation progressed intensively in the intact and in the hypophysectomized-STH-treated animals. In these groups, there were also certain qualitative changes in bone structure, which are quite characteristic of lathyrism. For example, within the newly formed periosteal bone and, sometimes, even in the fibrous, periosteal tissue not yet ossified, innumerable "basophilic bone globules" (Selye[6]) were found throughout the skeleton. Furthermore, many of the chondrocytes (which arise in lathyrism by metaplasia from periosteal connective tissue) became intensely basophilic. All these changes were greatly inhibited by hypophysectomy, but, in the STH-treated-hypophysectomized animals, they appeared to be even more pronounced than in the intact control rats (Figs. 6 to 10).

From these observations, it is evident that hypophysectomy markedly inhibited the development of bone lathyrism, but substitution therapy with STH alone sufficed to restore the reactivity of the bone to AAN. It is not yet clear why the lathyrism was even more pronounced in hypophysectomized-STH-treated rats than in the intact controls. The amount of STH given was certainly in excess of that normally secreted by the pituitary gland. Furthermore, hypophysectomy abolishes glucocorticoid and thyroid-hormone secretions and thereby reduces the blood level of substances known to inhibit lathyrism.

Summary

Hypophysectomy greatly diminished the development of bone lathyrism that is normally produced by aminoacetonitrile (AAN) in the rat.

Treatment with large doses of somatotrophic hormone (STH) restored the ability of the hypophysectomized rat to respond to AAN with the typical manifestations of bone lathyrism. In fact, under the conditions of these experiments, the lathyrism produced in hypophysectomized-STH-treated animals was even more severe than that produced by the same amount of AAN in otherwise untreated intact controls.

It is concluded that: (1) The normal hormonal secretion of the pituitary gland exerts a decisive influence upon the development of

bone lathyrism produced by AAN, and (2) a potent STH preparation will, in itself, suffice as a substitute for the ability of the pituitary body to maintain the sensitivity of the skeleton to a lathyrogenic compound.

We gratefully acknowledge generous supplies of aminoacetonitrile (AAN) from Dr. I. V. Ponseti, State University of Iowa, Iowa City, Iowa; Cortril (COL) from the Pfizer Laboratories, and Somatotropin (STH) from the Armour Laboratories.

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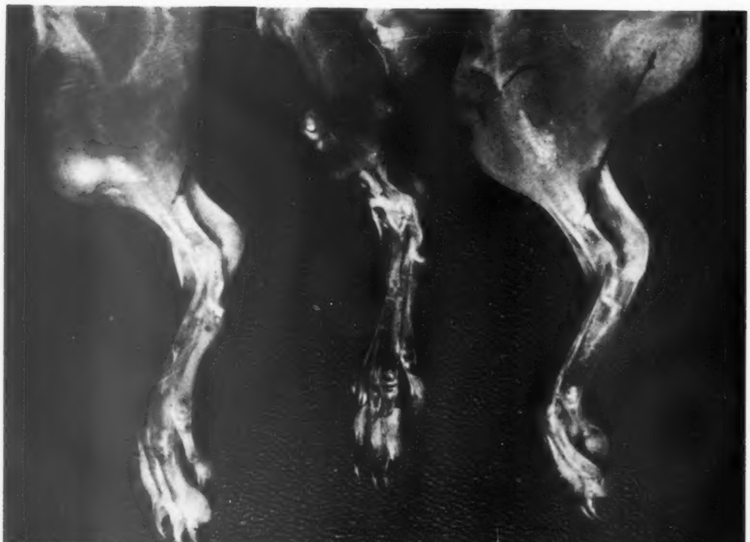
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Appearance of the tibial fractures after removal of the skin. In the intact (left) and in the hypophysectomized-STH-treated (right) animals, there is a great deal of hard, callus tissue, while in the hypophysectomized, otherwise untreated rat (middle), callus formation is deficient, but a hematoma has formed at the site of the fracture.

FIG. 2. Mandibles of the three rats shown in Figure 1. There are numerous exostoses at muscle-insertion sites (particularly around the condyloid and coronoid processes and at the angle of the jaw) in the intact rat. Bone proliferation is even more intense in the hypophysectomized-STH-treated animal, while in the hypophysectomized, otherwise untreated rat, exostoses are hardly visible.



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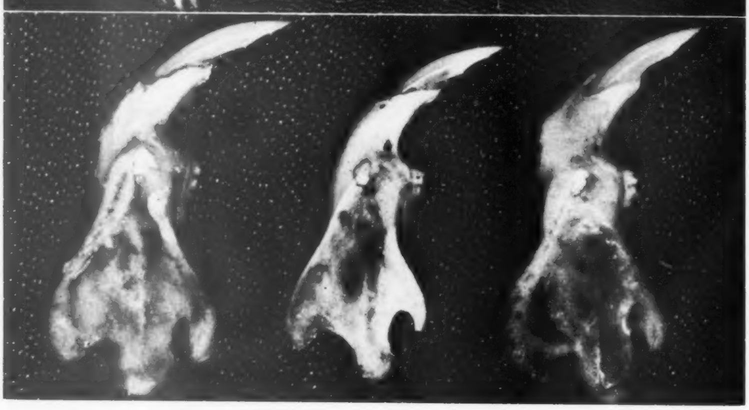

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FIGS. 3, 4, and 5. Histologic sections through the fractured tibias shown in Figure 1. The excessive fibrocartilaginous callus formation in the intact (Fig. 3) and hypophysectomized-STH-treated (Fig. 5) rats is in sharp contrast to the very slight callus formation in the hypophysectomized, but otherwise untreated rat (Fig. 4). × 5.

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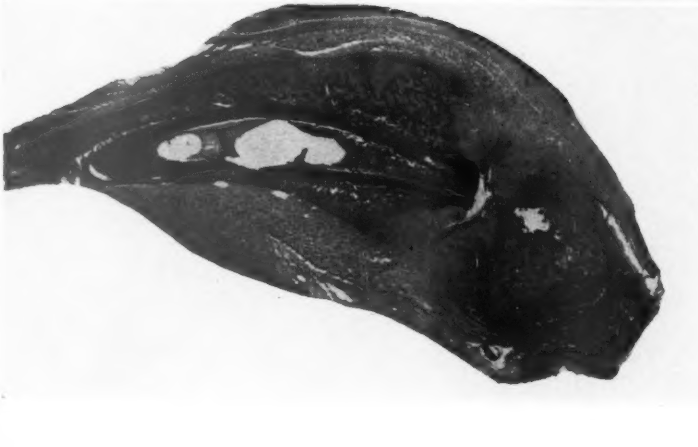

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FIGS. 6, 7, and 8. Typical regions of periosteal new-bone formation near the middle of the femoral shafts of the rats shown in Figures 3, 4, and 5 (periosteum to the left, marrow cavity to the right). Here, as in other non-traumatized skeletal parts, the excessive periosteal trabecular bone formation in the hypophysectomized-STH-treated animal (Fig. 8) is even more pronounced than in the intact control (Fig. 6), while in the hypophysectomized, otherwise untreated rat (Fig. 7) there is only some periosteal thickening, but almost no actual new-bone formation. × 20.

FIG. 9. A region from the femoral shaft illustrated in Figure 8, under higher magnification. The original compact bone of the shaft (lower margin) is clearly distinguishable from the adjacent cartilage which, in turn, is surrounded by partly ossified bony trabeculae. × 100.

FIG. 10. Still higher magnification from the section shown in Figure 9. Here the uneven basophilia (indicative of calcification) of the cartilage cell-capsules and the "basophilic bone globules" in the surrounding osteoid are clearly distinguishable. × 400.

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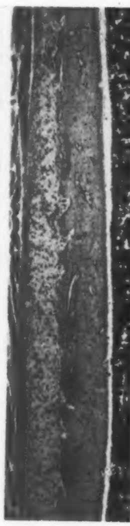

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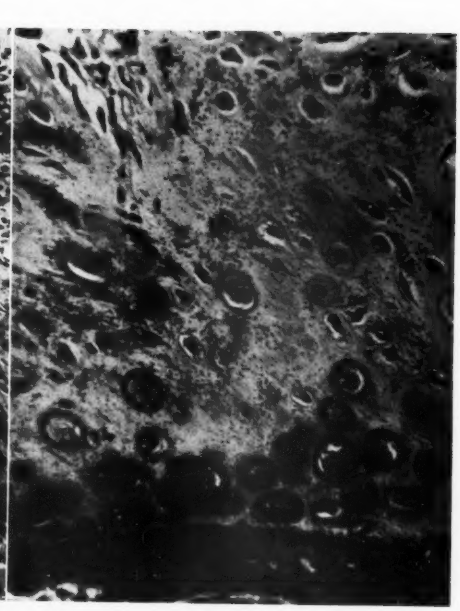

10

THE CHANGING CELLULAR DISTRIBUTION IN BONE MARROW OF THE NORMAL ALBINO RAT BETWEEN ONE AND FIFTY WEEKS OF AGE *

CHARLES HARRIS, M.D., and WILLIAM T. BURKE, B.A.
From the Samuel S. Fels Research Institute, Temple University Medical School, Philadelphia 40, Pa.

A study of the cellular composition of the bone marrow of albino rats between birth and 1 year of age was undertaken to provide a baseline of normal values with which the marrow composition of experimental animals in the same age group could be compared. It was found that the bone marrow undergoes a considerable change in composition during the first 10 weeks of life, and that the alterations are so distinctive that the age of any animal in our colony between 1 and 10 weeks can be deduced with considerable accuracy after careful study of smears of its bone marrow.

METHOD

All rats were derived from the original Wistar colony, and random bred at the Fels Institute for the past 7 years. Ten rats in each age group were studied. They were sacrificed either by decapitation or ether vapor. Sections of liver, spleen, and vertebral marrow were placed in Zenker's acetic acid solution and samples of blood and bone marrow were smeared and painted,[1] respectively, on glass slides. Dry smear preparations of each marrow and peripheral blood sample were stained either by the May-Gruenwald-Giemsa method or a benzidine-peroxide technique, that is essentially Lillie's (1952) modification of Washburn's method except that the slides after being fixed in formaldehyde vapor were first mordanted in 0.5 per cent copper sulfate. Values for the peripheral blood counts were derived from evaluations of 400 cells each, and those for marrow from counts of 1,000 cells, of both the May-Gruenwald-Giemsa and benzidine-peroxide preparations. In the peripheral blood smears, benzidine positive granulocytes (plus), faintly positive monocytes (plus-minus), and negative cells, lymphocytes, were easily distinguished. For marrow studies, the benzidine method served as a check on the true granulocyte count.

* Studies in this investigation were supported by research grant C-2206(2) from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

Presented at the Fifty-third Annual Meeting of the American Association of Pathologists and Bacteriologists, Cincinnati, Ohio, April 26, 1956.

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Values for the peripheral blood counts (Table I) have been translated into absolute figures for comparison. The percentage figures for marrows, however, are equivalent to an absolute value for the volume of marrow represented by the 1,000 cells counted, since no hemodilution of marrow occurs with the technique used, and on this basis the cellular composition of different marrow samples can be compared.

Although all counts were done from strips of marrow painted on slides and stained with the May-Gruenwald-Giemsa combination of dyes, the morphologic characteristics of individual cells were also studied from samples of bone marrow which were diluted with rat serum prior to making the smears. The admixture of the marrow with serum increased the affinity of the cells for the stain to such a degree that the Giemsa stock solutions had to be diluted from 5 per cent, which we found to be optimum for undiluted marrow samples, to about 2.5 per cent to prevent overstaining; nonetheless, the use of diluted marrow samples enhanced the clarity of the stain and resulted in crisper definition of minute structure.

Nomenclature

For purposes of classification we have used the following terminology.

The term *blasts* refers to a group of benzidine negative, agranular cells, 20 to 30 μ in diameter, with a thin rim of cytoplasm which is usually basophilic but occasionally amphophilic (Figs. 1D, 1E, and 2). The nuclear membrane is thin, nucleoli are often but not always present, and the chromatin is arranged in leptochromatic threads. Nucleoli were more prominent in the cells of marrow samples that had been mixed with serum before smears were made, than in the cells of undiluted marrow strips.

A cell similar to a blast except for the presence of a few azurophilic granules was termed a *promyelocyte*, whereas increase in granulation and the appearance of a few species specific granules were sufficient criteria to classify the cell as a *myelocyte* (Fig. 3). At this stage, a small nuclear vacuole would often appear, or the nucleus might assume an indented shape.

The typical *metamyelocyte* is an extension of these changes, showing either deepening of the hof or widening of the space within the nucleus to provide a ring-shaped nuclear mass (Fig. 4). Also included under the heading of metamyelocyte is a small cell about half again as large as an erythrocyte, with a nucleus in which clumping of chromatin is present. A few granules appear in these cells which are benzidine

positive. The nucleus may or may not contain a small vacuole or space. This cell resembles a cross between a lymphocyte and a granulocyte (Figs. 4, 13, and 14). It is not the typical metamyelocyte, but a different predecessor of some of the polymorphonuclear granulocytes and represents about the same stage of development in its category of granulocytopoiesis that the metamyelocyte represents in the classical myeloid derivations.

The category of *mature granulocytes* includes the stab form and indicates a stage of complete differentiation in which mitotic figures no longer occur. The nucleus forms a ring, the rim of which thins as the cell matures; at the same time the nuclear chromatin is arranged in thicker, more basophilic braids (Fig. 5). At this stage the cell is about 14 to 20 μ in diameter, contains specific granules, and, as do cells of the entire series starting with the promyelocyte, gives a positive benzidine reaction. This cell in marrow smears may be completely agranular and the cytoplasm may fail to take a stain. Other forms show slight granulation. The percentage of eosinophils and basophils is relatively constant throughout, and these cells have been grouped with the respective categories in the granulocyte series.

The *lymphocyte-like cell* group includes a few true lymphocytes, but is composed mainly of a small (8 to 12 μ) round cell with a thin rim of light blue, clear cytoplasm (Figs. 1A, 6, 7, and 8). The nucleus stains reddish umber and is composed of a homogeneous chromatin with only the slightest suggestion of blocking or clumping. It has a smooth, almost hyaline quality and never contains nucleoli or chromatin strands. This cell has been called a small myeloblast by Naegeli[2] (1900); a lymphocyte by Maximow,[3] Bloom,[4] Dominici,[5] and others; a primitive-free cell by Cunningham, Sabin, and Doan[6]; a hematogone by Kato[7] and others[8,9]; and in order to convey the fact that the nucleus differs slightly from that of the mature lymphocyte, we have called it the lymphocyte-like cell.

The nucleated red blood cells were grouped as one, regardless of whether they were mature or young forms.

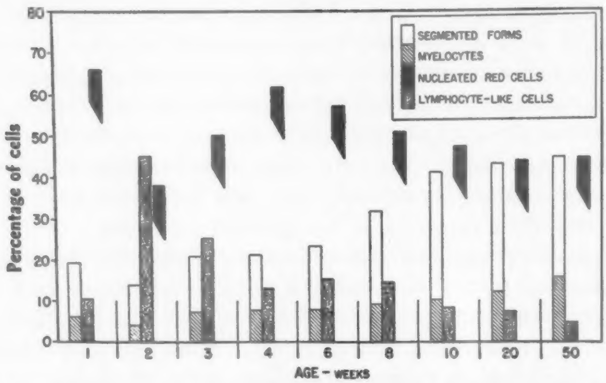
The *reticulum cell A* is generally large, round or occasionally stellate with lacy nucleoplasm containing widely spaced chromatin threads and is often granulated. The cytoplasm may be clear blue, or have a slightly opaque quality. A second form, *reticulum cell B* (Fig. 9), is oval with dark cotton-like blue cytoplasm and an eccentrically placed nucleus often appearing as if it were in the process of being extruded from the cell. It is found commonly in animals 1 and 2 weeks of age, but can be seen in animals up to 4 weeks of age. In paraffin sections

of bone marrow this cell is found lining bony spicules and may have osteoblastic function, but in smears it helps to identify the age of the rat since it has not been seen in normal animals after 4 weeks of age.

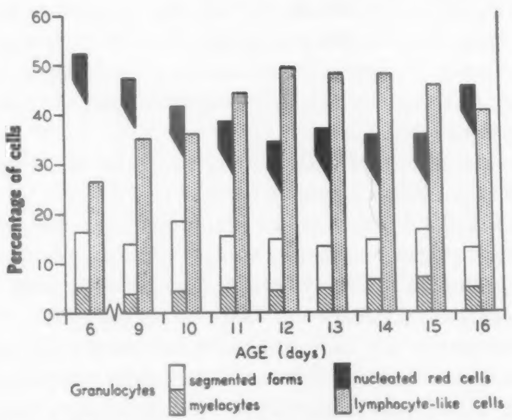
Plasma cells have the characteristic radkern nucleus placed eccentrically in a blue cytoplasm.

Results

As can be seen from Text-figures 1 and 2, marked changes in bone marrow composition occur between the first and third weeks of life, and somewhat more gradually between the ages of 6 and 10 weeks. The peripheral blood count changes only slightly during this period,



Text-figure 1. Change in the cellular composition of bone marrow of rats 1 to 50 weeks of age. Each datum represents the average of counts of ten animals.



Text-figure 2. Daily change in the cellular composition of bone marrow of animals of a single litter between the ages of 6 to 16 days. Each datum represents counts of a single animal of the litter.

TABLE I
*Average Determinations on the Peripheral Blood of Animals**

	1 Week	2 Weeks	3 Weeks	4 Weeks	6 Weeks	8 Weeks	10 Weeks	20 Weeks	50 Weeks
Hemoglobin (gm. %)	7.9 ± 0.6	8.2 ± 0.8	6.9 ± 1.6	8.6 ± 2.1	9.0 ± 0.3	10.6 ± 0.7	12.8 ± 0.2	13.1 ± 1.3	14.0 ± 0.7
White blood count (cells/cmm.)	4,600 ± 975	5,900 ± 952	6,685 ± 1,740	14,700 ± 8,700	7,000 ± 4,700	14,000 ± 7,000	11,200 ± 4,100	16,500 ± 3,300	13,500 ± 5,000
Segmented forms (%)	20.1 ± 7.0 (920)	22.7 ± 4.1 (1,357)	25.0 ± 15.0 (1,671)	13.7 ± 6.5 (2,058)	18.2 ± 7.6 (1,260)	14.3 ± 5.7 (1,960)	15.2 ± 8.2 (1,680)	16.3 ± 4.3 (2,640)	35.2 ± 8.1 (4,725)
Lymphocytes (%)	68.8 ± 10.8 (3,124)	72.8 ± 3.7 (4,248)	72.0 ± 14.8 (4,813)	73.1 ± 7.0 (10,731)	68.2 ± 8.4 (4,760)	77.8 ± 6.0 (11,060)	73.1 ± 10.3 (8,176)	75.9 ± 5.6 (12,540)	51.2 ± 9.9 (6,885)
Monocytes (%)	10.4 ± 7.6 (460)	3.0 ± 2.3 (177)	2.1 ± 1.4 (120)	13.1 ± 3.2 (1,916)	12.9 ± 3.9 (910)	7.1 ± 3.7 (980)	9.0 ± 2.8 (908)	7.2 ± 3.7 (1,155)	13.6 ± 2.1 (1,755)
Benzidine positive cells(%)	21.5 ± 5.6	15.4 ± 9.3	31.0 ± 0.7	15.5 ± 7.3	19.4 ± 7.9	14.2 ± 5.5	17.5 ± 7.3	19.7 ± 6.1	36.5 ± 7.6

* Averages derived from counts of 400 cells; 10 rats in each age group.
Numbers in parentheses represent absolute figures of differential count.

but the hemoglobin drops markedly between the ages of 2 and 3 weeks which may be related to the marrow changes at that time (Table I).

The marrow at 2 weeks is characterized by a sudden increase in nongranulated mononuclear cells, most of which are morphologically identical with the lymphocyte-like cell, except that they vary from 10 to 25 or 30 μ in size. Concomitantly, an increase in blasts occurs (Table II), and many forms are seen which "bridge" the morphologic gap between lymphocyte-like cells and blasts (Figs. 1, 10, and 11). A simultaneous and sharp decrease in nucleated red blood cells occurs. The myelocytes increase, metamyelocytes drop to about one fourth of their original value, and the number of mature granulocytes diminishes. These changes result in a slight drop in the total granulocyte count. By the third week the number of nucleated red blood cells has increased, the trend in the granulocytic series is reversed, and the tide of mononuclear cells is receding. At that time, the nucleated red blood cells show consistent uniformity in size, averaging 15 to 20 μ in diameter, and in many instances resemble the lymphocyte-like cells (Figs. 8 and 12). Text-figure 2 illustrates the day by day change in marrow composition during this period.

By the fourth post-natal week, this protean mononuclear cell, which we call the lymphocyte-like cell, again comprises 10 to 15 per cent of marrow cells and except for increased nuclear basophilia and condensation, is identical with its counterpart in the 1 week animal. It again is uniform in size, measuring 10 to 12 μ in diameter. The larger mononuclear cells have disappeared and blasts are again decreased in number.

Between the sixth and tenth post-natal weeks, there is an increase of granulocytes in the marrow from 20 to 40 per cent, which is accomplished mainly by the direct transformation of this small lymphocyte-like cell to a small ring-form of polymorphonuclear leukocyte (Figs. 1, 13, and 14). At first the nucleoplasm tends to condense and become "blocked." Then a small space appears which gradually enlarges, thinning the surrounding nuclear substance which seems to coil like a rope. At the inception of this change, often before a hole appears in the nucleus, the cytoplasmic border expands, and granules as well as a positive benzidine reaction develop.

By the eighth week the transition from lymphocyte-like cell to granulocyte is predominant and great difficulty was encountered in classifying the transitional cells because they had all of the characteristics of lymphocytes except that they contained cytoplasmic granules (Fig. 15). These cells were classified as metamyelocytes only because we believed that they represent a stage of development comparable to

TABLE II

*Average of Differential Counts made from the Femoral Bone Marrows**

Cells (%)	1 Week	2 Weeks	3 Weeks	4 Weeks	6 Weeks	8 Weeks	10 Weeks	20 Weeks	50 Weeks
Blasts	1.3 ± 0.6	2.1 ± 0.9	2.2 ± 0.7	1.0 ± 0.6	0.8 ± 0.5	0.7 ± 0.4	0.6 ± 0	0.9 ± 0.3	0.8 ± 0
Promyelocytes	0.2 ± 0.1	0.4 ± 0.2	0.5 ± 0.3	0.4 ± 0.5	0.2 ± 0	0.2 ± 0	0.2 ± 0.1	0.6 ± 0.3	0.6 ± 0
Myelocytes	1.5 ± 0.8	2.5 ± 0.6	4.2 ± 1.6	1.3 ± 0.6	2.1 ± 0.5	2.4 ± 0.1	2.5 ± 0.8	2.2 ± 1.1	3.9 ± 1.3
Metamyelocytes	4.7 ± 1.1	1.2 ± 0.4	2.6 ± 0.9	6.1 ± 1.4	5.9 ± 1.7	6.9 ± 0.5	7.4 ± 1.2	10.3 ± 2.3	11.5 ± 1.9
Stab cells and mature granulocytes	13.0 ± 2.9	8.4 ± 1.6	10.1 ± 2.3	13.6 ± 3.7	15.6 ± 3.3	22.5 ± 5.6	31.0 ± 7.6	30.1 ± 5.2	29.0 ± 2.6
Lymphocyte-like cells	10.8 ± 3.5	45.1 ± 4.5	25.3 ± 7.7	13.1 ± 3.2	15.4 ± 3.6	14.8 ± 5.0	8.4 ± 2.6	7.4 ± 3.5	4.9 ± 2.6
Plasma cells	0	0	0	0	0	0.1 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	1.3 ± 0.7
Reticulum cells A	0	0	0	0.2 ± 0.1	0	0	0	0.2 ± 0.1	1.3 ± 0
Reticulum cells B	0.4 ± 0.3	0	0	0	0	0	0	0	0
Nucleated red blood cells	66.0 ± 5.5	38.1 ± 3.2	50.2 ± 7.7	62.0 ± 5.4	57.5 ± 2.6	50.1 ± 0.8	47.4 ± 6.5	44.0 ± 3.9	44.8 ± 3.9
Total Granulocytes	19.4 ± 2.5	12.5 ± 2.5	17.4 ± 4.0	21.4 ± 4.5	23.9 ± 4.0	32.0 ± 5.5	41.1 ± 7.9	43.2 ± 4.7	44.5 ± 3.1
Benzidine positive cells	17.6 ± 4.6	15.7 ± 5.6	17.0 ± 4.4	19.4 ± 4.2	25.5 ± 4.1	30.8 ± 5.4	40.8 ± 4.6	42.8 ± 3.6	45.2 ± 3.8

* All bone marrow values derived from counts of 1,000 cells; 10 animals in each group.

that which the metamyelocyte represents in the myeloid transformation. Perhaps a better cognomen for these cells would be "lymph-polys" which more accurately describes their transitional nature. Thus, without undergoing mitosis, and without changing its size or shape, the lymphocyte-like cell undergoes a heteroplastic alteration to become a mature granulocyte.

The marrow of 8-week-old rats, when seen under high-dry magnification, shows a predominance among the leukocytes of small granulated (benzidine positive) cells, the nuclei of which contain small central spaces of increasing dimensions (Figs. 15, 16, and 18). This is in such marked contrast to the non-granulated mononucleosis which typifies the marrow at 2 weeks of age (Figs. 17 and 19), that the approximate age of the animal can be guessed from examination of the marrow smears.

The megakaryocytes of the marrow, polymorphonuclear and polynuclear varieties, were more plentiful in the younger (1 to 2 weeks) animals, but no counts of those cells were made. Plasma cells were never found in animals less than 8 weeks of age and, conversely, the large reticulum cell B, with oval fibrillar vacuolated cytoplasm having a cotton-like texture and with eccentrically placed nucleus, was found in clusters of four or five cells in animals 1 to 2 weeks of age (Fig. 9), singly in animals 3 weeks of age, and only rarely in the marrow of normal animals 4 weeks or older.

Two other changes worthy of note and related to those described above were: the sudden appearance of increased amounts of fat in the marrow of 2-week-old animals which disappeared only to return in similar quantity in animals 6 months to 1 year of age (Table III), and the recurrence of myeloid metaplasia in the spleens of animals 6 weeks of age, coincident with the heteroplastic transformation of lymphocyte-like cells to granulocytes in the bone marrow (Table IV).

Interpretation and Discussion

One of the main difficulties in studying cell lineage in bone marrow preparations is that under the homeostatic conditions that obtain in the normal mature animal, the cells most vital to the study, namely, transitional forms, are often present in too small a number to permit an observer to relate them to cells from which they derived, or to cells which derive from them. It is for this reason that the changes described above, although present, cannot be easily traced in the marrows of the normal adult rats. If only 2 per cent of cells undergo transition at any time, a smaller fraction of these will be in-between or "zwischen" forms. If only four or five cells in 1,000 represent

TABLE III

Fat Distribution in Bone Marrow of Normal Rats

Age of rat	Rat no. 1	2	3	4	5	6	7	8	9	10
1 Week	o	o	±	+	+	+	+	+	+	+
2 Weeks	+	+	+	+	+	+	++	+++	+++	+++
3 Weeks	o	o	o	±	+	+	+	+++	+++	+++
1 Month	o	o	o	o	o	o	o	+	+	++
6 Weeks	o	o	o	o	o	o	o	o	o	±
8 Weeks	o	o	o	o	o	o	o	o	o	++
10 Weeks	o	o	o	o	o	o	o	o	o	+
6 Months	o	o	o	o	±	+	+	++	++	+++
12 Months	+	+	+	++	++	+++	+++	+++	+++	+++

Marks, from + to ++++, indicate roughly the amount of fat present in large vacuolar form in smears of the femoral bone marrow of each rat in each group studied.

TABLE IV

Intensity of Myeloid Metaplasia in Rats One to Fifty-Two Weeks of Age in Liver and Spleen

Age of animal	Spleen	Red pulp: Erythrocyte content	Red pulp: Myeloid metaplasia	Liver: Myeloid metaplasia
1 Week	The malpighian corpuscles are small and have a homogeneous cellular composition; the mantle of light staining mononuclear cells is absent	o	++++	++
2 Weeks	The malpighian corpuscles are enlarging and in 30% of the animals, a mantle of light staining mononuclear cells can be observed	+	+++	+
3 Weeks	Continued enlargement of the malpighian corpuscles and a mantle of light staining mononuclear cells observed in 60% of the cases	++	++	±
4 Weeks	The malpighian corpuscles are prominent and 70% of the animals have a mantle of light staining mononuclear cells	+	+++	o
6 Weeks	The malpighian corpuscles are similar in size and appearance to those found in the mature animals, the mantle of light staining mononuclear cells is observed in 100% of the cases	±	++++	o
8 Weeks	Similar to 6 weeks	++	+++	o
10 Weeks	Similar to 6 weeks	++++	++	+
26 Weeks	Similar to 6 weeks	++++	+	o
52 Weeks	Similar to 6 weeks	+++	+	o

morphologic links between two cell forms, their significance will be overlooked.

In this study, however, we found two periods in the life of the young rat during which mass alterations in distribution of marrow cells occurred, so that at least half of the observed cells were in one phase of transition or another, from a primitive cell form to a more mature entity. In the first phase, at 2 weeks of age, the transition involved a series of mononuclear cell reactions that eventually resulted in the production of myeloblasts and normoblasts, and the second change between 6 and 10 weeks of age concerned the production of benzidine positive granulocytes from the lymphocyte-like small mononuclear cell. The number of blasts shows an increase at 2 weeks which might have been higher were not numerous bridge forms included in the lymphocyte-like column (Table II). Observation of the 3-week animals indicates a certain uniformity of size of normoblasts, and transitions between lymphocyte-like cells and normoblasts are easily found. Many enlarging lymphocyte-like cells, after reaching a diameter of about 20 μ, convert to normoblasts of the same size by gradually developing hyperchromatism of the nuclear and cytoplasmic elements (Figs. 8 and 12). By the third week a sudden increase in the number of myelocytes occurs, suggesting heteroplastic granulocytosis of many of the blasts.

The second change occurring between 6 and 10 weeks involves a metaplasia of the lymphocyte-like cell to a granulocytic form, a process that was called by Dominici[5] (1909) an amyloid transformation of the lymphocyte to the myelocytic series. In the adult rat, however, although these smaller bridge forms are observed, the predominant picture is the myeloid transformation through large myeloblasts.

All of the changes described above are illustrated in Figure 1, which details the marrow cell relationships in the baby rat.

The lymphocyte-like cell which plays so large a part in these transformations is the multipotential cell called a lymphocyte by Maximow,[3] Bloom,[4] and Dominici[5]; a hemoblast by Jordan and Kindred[10]; a small myeloblast (*vide infra*) by Naegeli[2]; a primitive-free cell as illustrated in their Figure 10 (1925) by Cunningham, Sabin, and Doan[6]; and hematogone by Kato,[7] Erf and Fine,[8] and Vogel and Bassen.[9]

In the article which first described the myeloblast, Naegeli[2] said that there is a small cell that is difficult to distinguish from a lymphocyte which increases in size to become the large myeloblast, and that in-between forms are numerous. He called the small and large mononuclear cells myeloblasts, but aside from this difference in nomenclature, his description could easily be used to describe the changes which

we observed in the marrow of 2-week-old rats. He noted that the small myeloblast is found most commonly in the younger age groups, and also mentioned that it has no nucleoli.

In 1931, Naegeli[11] ascribed definite qualities to the myeloblast, presumably including the micromyeloblast in this classification, but then added that "Eine weitere lymphoide Zellform *vor* dem Myeloblasten ist durchaus unbewiesen," and later, "Zwischen-formen zwischen Mesenchymzellen und Myeloblasten gibt es nicht." Our lymphocyte-like cell corresponds either to Naegeli's[2] (1900) small myeloblast which enlarges, or to the unproved zwischen form that falls between the embryonal stem cell and the myeloblast, to which we refer in the latter quotation.

Sabin's[12] comments on this cell are germane to the entire discussion of hematopoiesis, particularly in relation to the unitarian-dualist arguments. She said:

> . . . in normal bone marrow, it is the primitive free cell of small size, similar to the small lymphocyte, but differing from it in having fewer mitochondria and a less differentiated nucleus, which is to be found in the largest numbers. It is this small cell which has been confused with the mature small lymphocyte, which has been so great a stumbling block in the understanding of the mechanism of bone marrow. The reason for this difficulty is clear, for the morphological points of distinction between the primitive free cell and the small lymphocyte are not striking; it is now certain that lymphocytes may arise in bone marrow both under normal and under pathological conditions, but the usual cellular output of the marrow is limited to red cells and the three strains of the granulocytic leucocytes, so that the primitive free cell of bone marrow normally produces granulocytes in that location.

The direct transformation of these cells to granulocytes in such large numbers at the age of 6 to 10 weeks is a change identical to that observed by Dominici[5] in rabbit omentum, by Danchakoff[13] when she implanted spleen of the adult hen on chick allantois, by Bloom[4] in the lymph node of guinea pigs subjected to anaphylactic shock, by Pappenheim, Maximow, Weidenreich, Downey,[14] and many others. Until the present, however, the various authors had to invoke different systems and experimental techniques as well as pathologic leukemic tissue as a source for cell derivations, and this varied source of experimental material has further confused the problem. In this study, however, all of the changes described in the literature are found to occur normally; i.e., the growth of lymphocyte-like cells to large blasts which transform themselves to cells of the red and white series at the ages of 1 to 3 weeks, and the direct myeloid transformation of lymphocyte-like cells to small granulocytes at 6 to 10 weeks. These two changes occur as an orderly response to separate biologic influences, probably extracellular in nature, which become dominant at the second and eighth weeks of life, respectively.

The changes observed by both unitarians and dualists are identical. The two groups argue mainly about interpretation; i.e., whether the small marrow cell is a micromyeloblast, a hematopoietic cell indigenous to marrow, or whether this cell is a multipotential lymphocyte. We agree with Ferrata[15] that the myeloblast transformations can be by-passed entirely under certain conditions. The importance of the myeloblast theory is that it presupposes the separation of two cellular systems, presumably independent of each other—the lymphatic and the myeloid—the one able to attain ascendency only at the expense of the other. What seems most probable, however, is that the bone marrow provides a milieu favorable to myeloid proliferation and that in the normal animal the lymph nodes and spleen provide a milieu favorable to lymphocytic proliferation. In early postnatal life and under certain experimental conditions the spleen can produce granulocytes and red blood cells, usually by direct myeloid transformation of the small lymphocyte to a granulocyte or a nucleated red blood cell of similar size, whereas in the 2-week-old rat a similar mononuclear cell reaction is made possible in the bone marrow by a sudden change in external chemical environment, the nature of which is unknown. It is not necessary to think of two different systems of cells, therefore, but of different extracellular influences, which cause differentiation in one direction or another at whatever site they happen to be exerting their influences. Hypothetically, the two humoral influences should not be able to operate simultaneously at the same site. It is of interest that extensive myeloid metaplasia recurs in the spleen at 6 weeks of age, at the same time that the marrow is starting to undergo a similar process.

The question of the myeloblast must diminish in significance because, far from being the only pathway of granulocytopoiesis, it is only a link in an occasional pathway of hematopoiesis, and can be bypassed in many instances by more direct transformations.

One should seek solution to the mystery of the hematogone, micromyeloblast, lymphocyte, or lymphocyte-like cell in the changing cellular composition of the marrows of the infants rather than the adults of various species. That this may hold true in man as well is indicated by the enlarging body of data which shows an increase in marrow "lymphocytes" up to 50 per cent of the total number of cells during the first year of life.

Summary

The distribution of cells of bone marrow of the albino rat undergoes two changes during the first year of life. In both instances the change is related to morphologic and numerical alterations in a distinctive

mononuclear cell that resembles a small lymphocyte and normally accounts for 5 to 10 per cent of the marrow cells.

At 2 weeks of age, this cell, having undergone variation in size so that all diameters between about 12 and 20 μ are represented, comprises 40 per cent of marrow cells. As the cell increases in size, the chromatin becomes more lace-like and the cell cannot easily be differentiated from blast forms.

This reaction subsides during the third week of life and the marrow differential count of animals 4 weeks of age is similar to that of animals 1 week of age. The differential count then remains relatively constant until 6 weeks of age when the lymphocyte-like cell undergoes transformation of another type. Nuclear chromatin becomes less hyaline and more clumped; azurophilic and benzidine positive granules appear; a hole appears in the nucleus and the cell can no longer be differentiated from the ring form of the polymorphonuclear leukocyte, a form common to the rat. It is this transformation that accounts for the increase of granulocytes of rat marrow from 20 to 40 per cent of the total number of cells in animals 6 to 10 weeks of age.

We wish to thank Mrs. Grace Brotherston and Mrs. Diana Giusti for their technical assistance.

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LEGENDS FOR FIGURES

FIG. 1. The heteroplastic transformations that occur in the cells of the bone marrow of young rats are illustrated. At 2 weeks of age the predominant pathway of cellular development originates with the lymphocyte-like cell (A) which undergoes considerable increase in size to become a typical blast cell. This alteration is illustrated in the progression of cells A, B, C, D, and E; during the following week, many of these cells undergo the transformation illustrated in cells A, F, K, L, and M, to become normoblasts. At 8 weeks of age, about 50 per cent of the marrow becomes involved in the heteroplastic transformation of lymphocyte-like cells to granulocytes as illustrated by cells A, F, G, H, I, and J. The lymphocyte-like cell (A) is also called lymphocyte, micromyeloblast, hemoblast, primitive-free cell, and hematogone, by various authors.

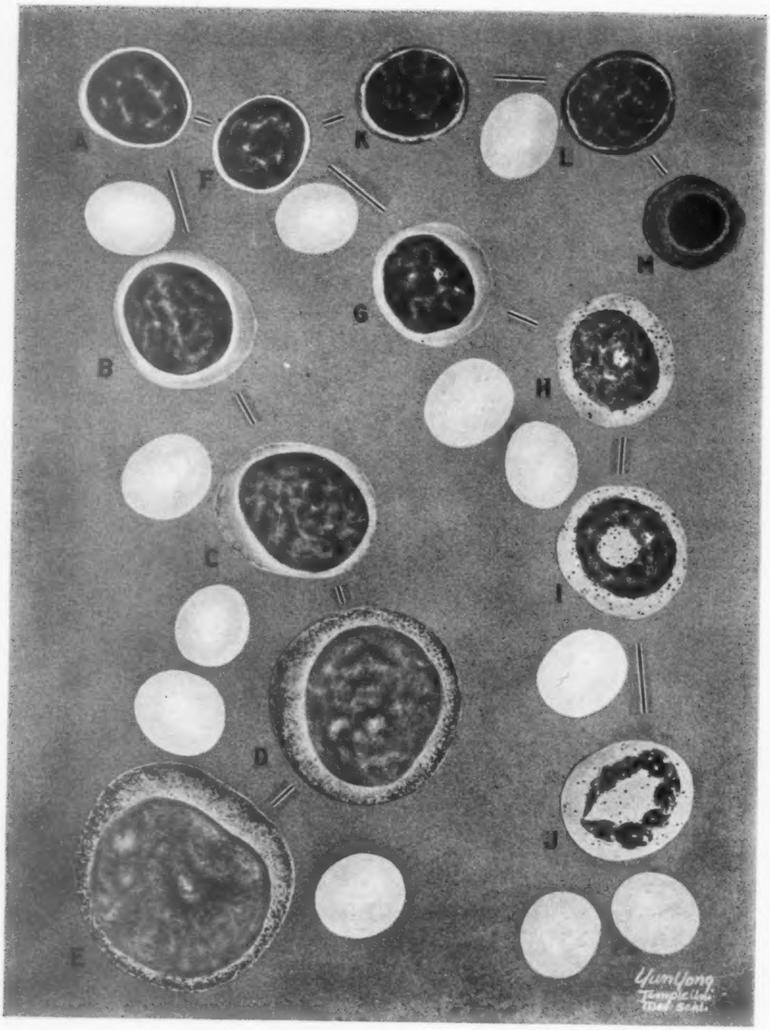

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FIG. 2. Blast cell adjacent to normoblast. × 2,500.

FIG. 3. Young myelocyte with azurophilic as well as species specific granules. × 3,000.

FIG. 4. Two cells which have been classified as metamyelocytes in Table II. The larger is derived from a myelocyte as portrayed in Figure 3. The smaller develops directly from a lymphocyte-like cell, and has fewer granules. × 1,600.

FIG. 5. Mature granulocytes of various types. Of note is the paucity of granules. × 1,600.

FIG. 6. Cluster of lymphocyte-like cells exhibiting the hyaline nuclear quality so often characteristic of this cell. × 1,600.

FIG. 7. Lymphocyte-like cells exhibiting variation in size. × 1,600.

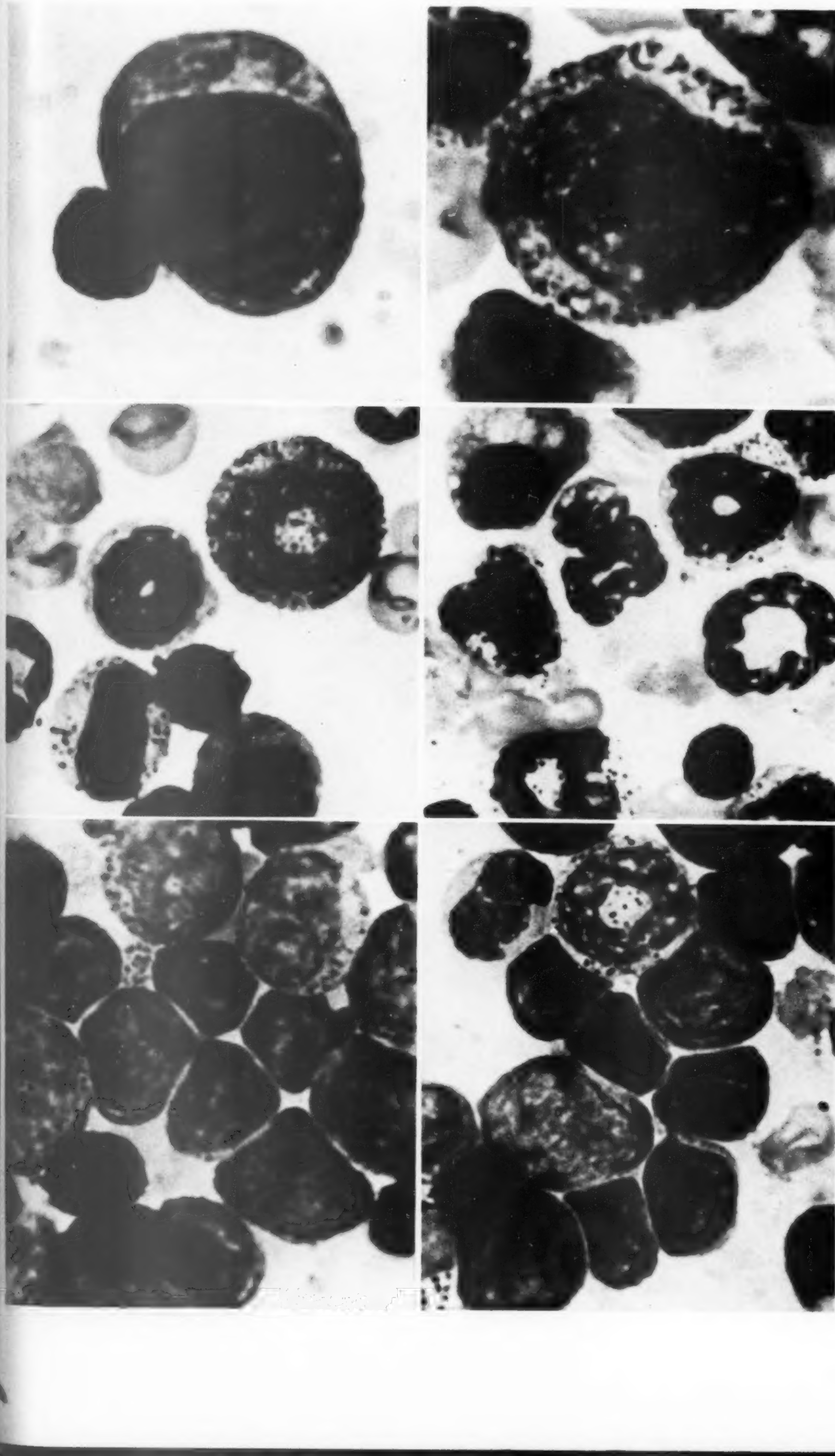

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FIG. 8. Lymphocyte-like cells exhibiting variation in size undergoing morphologic transition toward the red blood cell series at arrows. × 1,600.

FIG. 9. Reticulum (B) cells. On marrow smears the nuclei of these cells often appear partially extruded. These may be osteoblasts, but seem to identify the age of young rats because they are not normally seen in marrow smears of rats 4 weeks or older. × 800.

FIG. 10. Lymphocyte-like cells exhibiting transition to blast forms. × 1,600.

FIG. 11. Lymphocyte-like cells from spleen during phase of myeloid metaplasia, exhibiting transition to large lymphocyte forms. Of note, in comparison with Figure 10, is the similarity to the blast form. × 1,600.

FIG. 12. A nucleated red blood cell is adjacent to a lymphocyte-like cell, from which it can be seen to derive in marrow of rats 3 weeks of age. × 1,600.

FIG. 13. Lymphocyte-like cell showing some clumping of nuclear chromatin, as well as early granulation. The adjacent cell is in a more advanced stage of metaplasia toward mature granulocytes. × 2,500.

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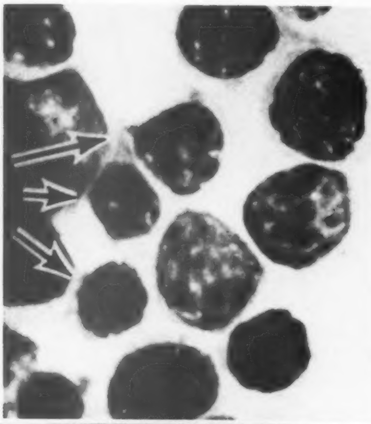

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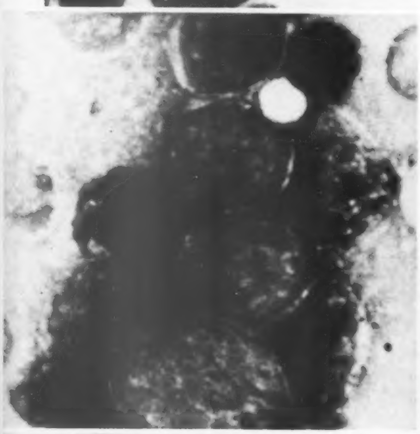

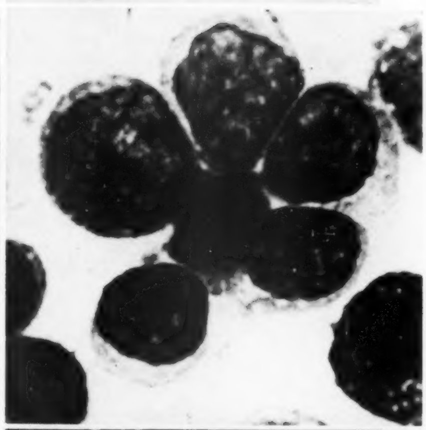

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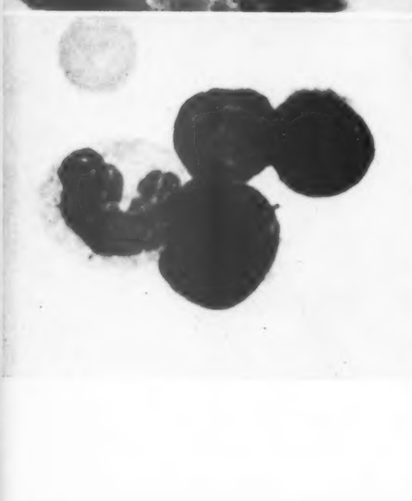

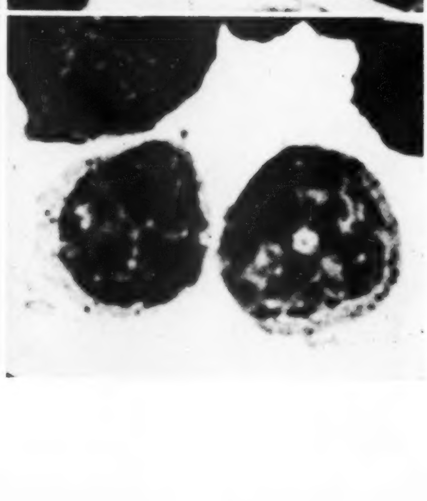

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FIG. 14. Another example of the metaplasia of lymphocyte-like forms to granulocytes. × 2,500.

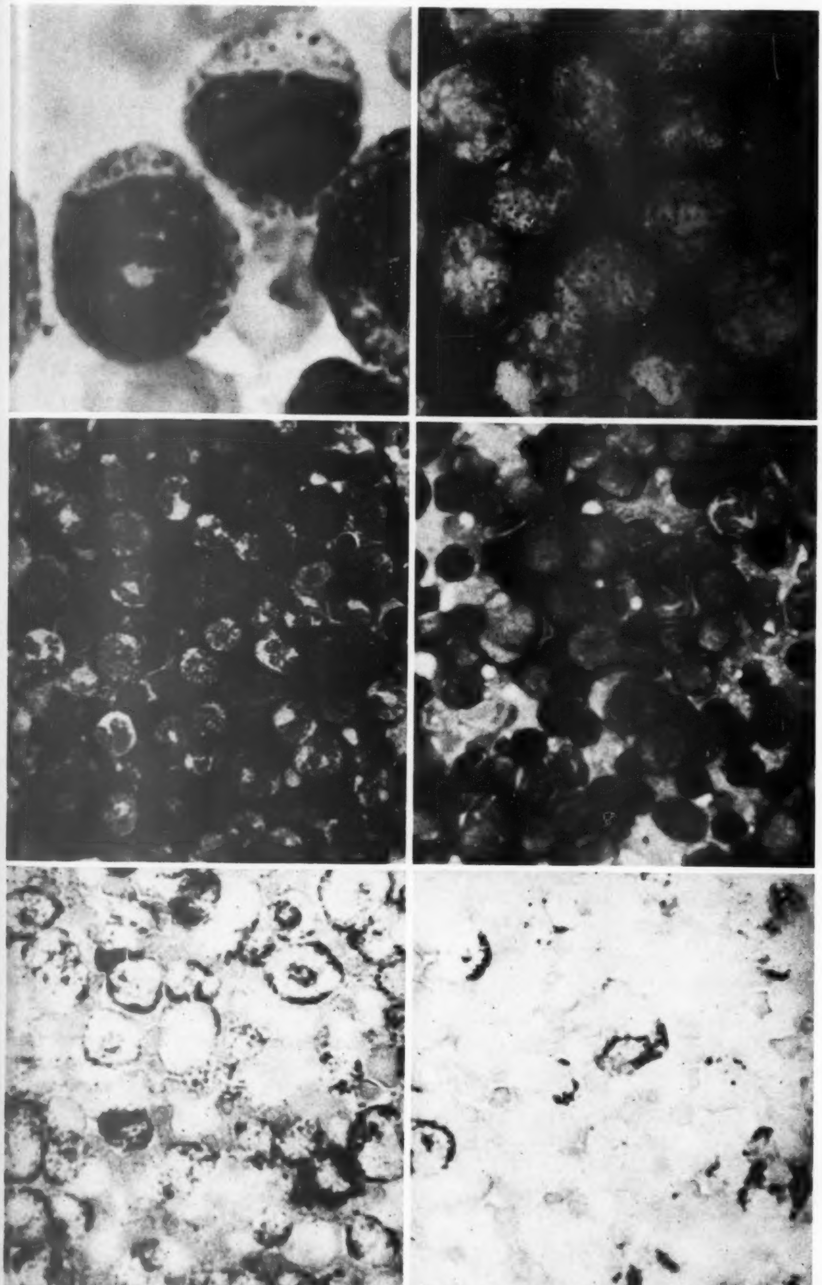
FIG. 15. Cluster of small "lymph-polys" prior to definite identification as cells of the granulocyte series. × 1,600.

FIG. 16. A typical example of bone marrow of an 8-week-old rat containing numerous small, granulated cells which have just undergone heteroplastic transformation from lymphocyte-like cells to granulocytes. × 800.

FIG. 17. Typical example of marrow from a rat, 2 weeks of age, exhibiting many non-granular lymphocyte-like cells. For comparison with Figure 16. × 800.

FIG. 18. Benzidine stain of marrow taken from an 8-week-old rat. For comparison with Figure 19, × 800.

FIG. 19. Benzidine stain of marrow taken from a 2-week-old rat. For comparison with Figure 18. × 800.



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GASTROINTESTINAL GANGLIONEUROMAS

Brief Review with Report of a Duodenal Ganglioneuroma*

Elmer V. Dahl, M.D.†; John M. Waugh, M.D., and David C. Dahlin, M.D.
From the Sections of Surgery and Surgical Pathology, Mayo Clinic and Mayo Foundation,‡ Rochester, Minn.

Ganglioneuromas are relatively uncommon, but not rare, tumors composed of ganglion cells and nerve fibers. They are preponderantly benign, containing mature-appearing ganglion cells. Undifferentiated or incompletely developed cells are present in about one fourth of the lesions, and such tumors are prone to malignant behavior, including metastasis.[1] Since the report of the first well authenticated case, that of Loretz[2] in 1870, more than 300 ganglioneuromas have been described in the literature. These case reports have been collected and reviewed from time to time; 47 such cases were found by Wahl[3] (1914), 94 by McFarland[4] (1931), 171 by Raška and Škorpil[5] (1936), and 243 by Stout[1] (1947). Published reports of about 75 additional ganglioneuromas have appeared since Stout's review.

These tumors are found most frequently along the sympathetic ganglion chains. The next common site of origin is the adrenal glands. While ganglioneuromas have been described arising in nearly every organ of the body, examples from the alimentary tract are extremely uncommon. We have found reports of only 11 such cases, exclusive of several such tumors in the pharynx and tongue. Five of these 11 tumors involved the appendix, two were situated in the terminal portion of the ileum, two in the stomach, and two involved a segment of intestine that included the terminal part of the ileum, the cecum, and the ascending colon (Table I). The report of the gastric lesion described by Dupuy[17] and listed as a ganglioneuroma by Raška and Škorpil[5] is not convincing, especially since an illustration was not provided. The present report is, to our knowledge, the first description of a ganglioneuroma in the duodenum.

Report of Case

A 49-year-old white housewife came to the Mayo Clinic in May, 1956, for removal of a polypoid duodenal lesion. Her previous personal history was not pertinent, and there was no history of familial disease. Approximately 6 weeks previously, she had become aware of mild abdominal distress, described by her as

* Received for publication, December 24, 1956.

† On assignment from the U.S. Air Force.

‡ The Mayo Foundation, Rochester, Minnesota, is a part of the Graduate School of the University of Minnesota.

TABLE I
Reported Cases of Gastrointestinal Ganglioneuromas

Author	Year	Age and sex	Symptoms, clinical findings or preoperative diagnosis	Organ involved	Pathologic findings	Accompanying disease or defects and remarks
Oberndorfer[6]	1921	28 M	Acute perforating appendicitis	Appendix	Perforated, diffusely enlarged appendix 16 cm. long, with relatively uniform hypertrophy of entire wall; neurofibromatous proliferation in meso-appendix and all coats of appendiceal wall; numerous ganglion cells especially in submucosa, but also as far out as meso-appendix	Dermal tumors, cutaneous pigmentation, low intelligence; mother and brother had Recklinghausen's disease
Schultz[7]	1922	57 M	Incidental finding at necropsy	Appendix	Thickened, hard appendix, with increased muscularis and submucosa; numerous ganglion cells, nerve fibers, and Schwann's cells in submucosa, muscularis, and meso-appendix	Death due to gastric carcinoma
Lichtenstein and Ragins[8]	1937	8 F	Acute appendicitis	Appendix	Appendix normal in size; mucosa in distal third diffusely thickened, projecting into lumen; increased stroma with numerous ganglion cells	
Martinez-Gutierrez[9]	1943	18 F	Abdominal pain for 4 months; pelvic mass	Appendix	Appendix 45 cm. long, 3.6 cm. in diameter; "neurinomatosis" of appendix and adjacent mesentery; muscularis hypertrophied; greatly increased ganglion cells in submucosa, muscularis, and mucosa	
Masson and Branch[10]	1945	34 F	Appendicitis	Appendix	Appendix 13 cm. long, 4.5 cm. in diameter, with walls to 2 cm. thick; hypertrophy of muscle layers; greatly increased ganglion cells of intrinsic plexuses; neurofibromatosis of adjacent mesentery	
Baltisberger[11]	1922	50 F	Peritonitis due to strangulated inguinal hernia	Terminal ileum	Large plexiform neuroma in mesentery of ileum, with increased thickness of wall of adjacent 48 cm. of ileum; increased ganglion cells in plexuses and along nerve trunks into mesentery	Numerous, soft, cutaneous tumors; horseshoe kidney; postoperative death; ganglioneuroma not in herniated intestine
Poate and Inglis[12]	1928	30 M	Flatulence, fullness, epigastric discomfort, sluggish bowels for 6 years	Terminal ileum, cecum, ascending colon	Confluent ganglioneuromatous tumors, 0.5 to 10 cm. in diameter, projecting into intestinal lumen; intervening cecal wall to 1 cm. thick; tumors believed to arise in region of Meissner's plexus	"Cystic retroperitoneal tumor" resected 8 years previously; chronic duodenal ulcer found at operation
Jentzer and Fatzer[13]	1937	17 F	Enlarging abdominal mass for 2 to 3 years, ascites	Terminal ileum, appendix, cecum, ascending colon	Plexiform neuroma with numerous ganglion cells in diffusely thickened intestinal wall; large neurofibroma and spindle cell sarcoma in adjacent mesentery	Low intelligence, oxycephaly, dilated cerebral ventricles, palatum ogivale; postoperative death
MacMahon and Davies[14]	1945	16 F	Loose bowels, nearly daily abdominal pain and vomiting since infancy; abdominal mass	Terminal ileum	Terminal ileum (22 cm.) enlarged to 5 cm. in diameter, with increase of all elements in wall; numerous ganglion cells in all layers; large nerve trunks in adjacent mesentery	
Bertini[15]	1936	66 M	Gastric ulcer	Stomach, anterior wall	Smooth, pedunculated, 3 cm. tumor containing ganglion cells, sympathoblasts, and neurofibrils	Lesser-curvature ulcer, not in proximity of ganglioneuroma
Pitts and Hill[16]	1947	68 F	Abdominal distention, eructation for 3 years; one episode of ...	Stomach, pyloric antrum	Smooth, discrete, ulcerated, 5 cm. tumor containing ganglion cells, apparently not involving muscularis propria, projecting into gastric lumen	

"bloating and swelling" of the abdomen and "heaviness" in the epigastrium. The discomfort occasionally was present when she awoke in the morning. It was relieved when she drank milk or ate, and it continued, but in diminishing severity, until the time of her admission to the clinic. Two weeks after the onset of these symptoms, she had fallen from a horse and fractured her left seventh rib. While recovering, with her thorax strapped, she had noted soreness in the left midabdomen. This discomfort disappeared spontaneously within 1 week. Roentgenologic examination shortly after the onset of these symptoms had disclosed a small polypoid tumor of the middle third of the second part of the duodenum.

General examination at the clinic disclosed no pertinent abnormalities. Roentgenologic studies confirmed the presence of the polypoid lesion of the duodenum (Fig. 1).

Laparotomy revealed a sharply circumscribed, firm, approximately spherical nodule, 12 mm. in diameter, covered except at the base by freely movable mucosa. The lesion projected from the medial wall of the duodenum into the lumen at a point 2.5 cm. proximal to the ampulla of Vater. A red, granular dimple, 2 mm. in diameter, in the otherwise smooth overlying mucosa suggested superficial ulceration. The lesion was separated from the apparently normal muscularis propria by sharp dissection. Grossly, the tumor resembled a leiomyoma because of its uniform, pale, yellowish pink color, smooth external surface, slightly whorled cut surface, and firmness.

Study of fresh frozen sections revealed large and small ganglion cells, occurring singly and in clusters, in a fibrillar and connective tissue stroma. A diagnosis of ganglioneuroma was made. The duodenal defect was closed without further resection. The patient was asymptomatic 4 months later.

Histopathologic Appearance

The bulk of the duodenal tumor was composed of non-myelinated nerve fibers coursing in all directions but not forming any conspicuous nerve bundles enclosed by perineurium. Many of the fibers were accompanied by Schwann's cells, but there was no proliferation of these cells. A thin capsule containing some collagenous connective tissue surrounded the tumor, separated it in most places from the overlying muscularis mucosae, and merged with the mass of the tumor. A single large cluster of Brunner's glands remained relatively undistorted within the tumor tissue. The muscularis mucosae was irregular in thickness and direction; it was duplicated in some places and discontinuous in others. It was heavily infiltrated by inflammatory cells and partially destroyed beneath a small mucosal ulcer (Fig. 2).

The number of ganglion cells varied greatly from place to place in the tumor (Figs. 3 and 4). Most cell bodies were arranged in clusters. Extremely few nerve fibers ran through these cellular aggregates. The largest ganglion cells occurred singly. The majority of ganglion cells of all sizes were multipolar, and none were pigmented. Chromophilic substance could not be demonstrated in their cytoplasm by staining with toluidine blue and cresyl violet. Their nuclei were vesicular, had little apparent chromatin, and contained prominent nucleoli. Occa-

sional cells had two, less commonly three, nuclei (Fig. 5). The nuclei of the smaller ganglion cells stained deeply with hematoxylin, but these cells also had abundant cytoplasm and often had well developed nerve processes. Mitotic figures were not found.

The mode of origin of most of the processes from the ganglion cells, in smooth continuity with the cytoplasm of the cells, suggested their dendritic nature. These silver-impregnated fibers frequently branched but rarely did so in close proximity to the cell body. The fibers varied in thickness but were nearly all less than 1 μ in diameter as measured by an ocular micrometer. Occasional nerve fibers, usually arising more abruptly, had twice that diameter. Beading or the formation of gemmules was encountered in extremely few fibers. As many as five or six fibers could be traced from a single cytoplasmic protrusion in some of the large ganglion cells. This feature may aid in explaining the apparent disparity in the number of ganglion cells and the fiber mass. None of the ganglion cells were surrounded by capsular or satellite cells. An occasional small cell with irregular cytoplasm, closely applied to the body of a ganglion cell, might be interpreted as a satellite cell (Fig. 6).

A few ganglion cells were found between the separated strands of the muscularis mucosae, and an occasional ganglion cell was present in the mucosa proper. An increase in nerve fibers within the mucosa was not evident. Degenerative changes in the ganglion cells, including cytoplasmic fraying and vacuolation, and frank cellular disintegration, were frequent, especially in the larger cells.

In the mucosa at one margin of the tumor, occupying a region approximately 2 mm. in greatest dimension, were a number of unusual gland-like structures. These extended from within the duplicated misshapen muscularis mucosae into the mucosal stroma to the tips of the crypts of Lieberkühn (Fig. 7). They were formed by oval to elongate, sometimes irregular cells with a homogeneous, usually abundant, eosinophilic cytoplasm. The nuclei of these cells were uniform, small and round to oval, containing small, deeply stained nucleoli. The cytoplasmic outlines were indefinite. In the more slender cells, the nuclei were near one end. These cells occurred in aggregates often suggesting an acinar arrangement. Their derivation from the duodenal glands did not appear probable. The cells did not contain mucus or any demonstrable intracytoplasmic granules. Small ganglion cells were associated intimately with some of the aggregates. Unfortunately, the pertinent region lay at the extreme margin of the specimen, where the tissue had been distorted, and adequate characterization of these structures was difficult. Sections from this zone were submitted to four surgical pathologists, whose comments included

"reminiscent of carcinoid," "perhaps aberrant pancreas," and "hamartomatous." All agreed that these structures did not appear to be an unusual arrangement of immature cells of the neurogenic series and that their precise classification apparently was impossible.

Comment

According to prevailing opinion, ganglioneuromas are true embryonic tumors in that they are considered to represent proliferation of ganglion cells from retained undifferentiated neural elements that have migrated to normal or abnormal locations from the neural crest or primitive brain or spinal cord. These cells are assumed never to have attained complete adult differentiation.[18,19]

The 12 tumors forming the basis of this report (the one just described plus the 11 in Table I) can be divided into two groups. The two ganglioneuromas from the stomach and the one from the duodenum were sharply delimited, not involving the muscularis propria. These three tumors had no large nerve trunks or neurofibromatous changes in the adjacent tissues, nor was there local or segmental hypertrophy of the wall of the affected viscus.

The group of nine tumors arising more distally in the gastrointestinal tract were much less definitely circumscribed. All but two of these lesions had more or less symmetric hypertrophy of the various layers of the affected intestinal wall, leading sometimes to a description of gigantism of the intestinal segment. These changes were least prominent in Lichtenstein and Ragins'[8] case and in Schultz's[7] case and were dramatic in most of the others. Eight of the nine tumors exhibited enlarged nerve trunks, plexiform neuromas, accumulations of Schwann's cells, or neurofibromatous proliferation intimately associated with the tumors, usually ramifying within the adjacent mesentery as well as in the entire thickness of the intestinal wall. Greatly increased numbers of ganglion cells were noted in each case. These were most prominent in the region of Meissner's plexus in eight of the nine cases but often were noted also in the region of Auerbach's plexus, in the mucosa, less commonly in the serosa, and occasionally even along nerve trunks in the adjacent mesentery.

The increase in thickness of the intestinal wall was brought about by hypertrophy of muscular and connective tissue elements and by proliferated nerve fibers, Schwann's cells, and ganglion cells. In the patients described by Jentzer and Fatzer[13] and by Baltisberger,[11] proliferation of nerve fibers and Schwann's cells within the mesentery formed the bulk of the tumor.

The relationship of the nine lesions under discussion to the neural

and muscular proliferation in the appendix described by Masson[20] has not been established. Whether such tumors as these nine should be called ganglioneuromas is debated. Pick[21] and Pick and Bielschowsky[22] objected to the applications of this term to the lesions described by Schultz[7] and by Oberndorfer[6] and to a similar tumor found by Pick in a horse. Pick and Bielschowsky preferred to think of these growths as resulting from a developmental aberration in which a defect in the intestinal anlage led to true hypertrophy of the affected intestinal segment in combination with local neurofibromatosis. Two of the nine patients, those described by Oberndorfer and by Baltisberger, had manifest stigmas of Recklinghausen's disease, and the former gave a family history of neurofibromatosis as well. Several of the patients had developmental anomalies, as listed in Table I.

The association of ganglioneuromas and neurofibromatosis has been recognized for a long time,[1,23-26] as has that of ganglioneuromas and developmental abnormalities.[23,27] Regardless of how one chooses to consider these growths in the intestine, they do contain ganglion cells in excess, and ganglioneuroma appears at present to be a good category for them. They have been listed as such by others.[1,5] A case reported by Montgomery and O'Leary[28] may be mentioned here. Multiple cutaneous nodules that proved to contain ganglion cells developed in a 26-year-old man who was under observation for another disease. His appendix, removed 1 year before onset of the cutaneous lesions, contained considerably increased numbers of ganglion cells, chiefly in Auerbach's plexus. The patient's colon was incompletely rotated. The cutaneous nodules subsequently underwent involution.[29]

Several features of the tumor described in the present report might be interpreted as indicating anomalous development, in part, of the lesion. The absence of submucosal glands, excepting a single, large, undistorted, glandular cluster retained within the tumor, the irregularities in the muscularis mucosae, not adequately explained on the basis of compression by tumor, and perhaps the peculiar unexplained structures in one region of the mucosa might be considered developmental errors. Nevertheless, the stigmas of proliferative activity of the ganglion cells, the occasional binucleated and trinucleated cells, the variation in cellular size, and the increased density of nuclear chromatin in the smaller cells, with their abnormal arrangement in loose clusters (Fig. 4), speak adequately for neoplastic activity. Although no one has been so bold as to favor the concept of dedifferentiation of ganglion cells in the natural history of these lesions, both Zimmermann[26] and Stout[1] have hinted strongly at it. Such dedifferentiation remains a possibility in the inception of some ganglionic tumors, especially since good evi-

dence exists that adult sympathetic ganglion cells are not absolutely post-mitotic.[30]

Ganglioneuromas may be considered to be part of a continuous spectrum with regard to malignancy. Completely undifferentiated, highly malignant neuroblastomas stand at one end of this spectrum, and quiescent mature ganglioneuromas are at the other. Metastatic lesions from tumors with the degree of cellular maturity present in the duodenal lesion have not been described. Classically, ganglioneuromas exhibit histologic evidence of malignancy in one of two ways.[1] They may be composed of diffusely intermixed cells in various stages of immaturity and mature ganglion cells, or they may contain completely neuroblastomatous regions in a tumor that in other parts has only mature ganglion cells. Two of the 12 tumors from the gastrointestinal tract presented malignant histologic features. The polypoid gastric lesion described by Bertini[15] contained intermixed, small, darkly stained cells interpreted as sympathoblasts. Neither metastasis nor invasion of the adjacent gastric wall apparently occurred, although follow-up was not available. The tumor described by Jentzer and Fatzer[18] was not malignant in the classic fashion but was associated with a spindle cell sarcoma arising in a zone of neurofibromatous proliferation in the mesentery adjacent to the ganglioneuroma in the intestinal wall. The authors' illustration showed a neoplasm that may have been a malignant schwannoma. Both Oberndorfer[6] and Schultz[7] described syncytial cells in the lesions reported by them; they considered these syncytia to represent intermediate forms in the development of ganglion cells. Pick and Bielschowsky,[22] who subsequently reviewed sections from these tumors, considered these syncytial cells to be proliferating Schwann's cells. With the exception of Jentzer and Fatzer's case, none of the 12 ganglioneuromas from the gastrointestinal tract were clinically malignant, and none recurred in the stated follow-up period, which was usually short. Two of the patients died in the immediate postoperative period (Table I).

There are no specific signs or symptoms to point to a preoperative diagnosis of ganglioneuroma of the alimentary canal. The clinical histories and findings in three of the five patients who had appendiceal lesions led to a preoperative diagnosis of appendicitis. One of the appendices was ruptured, and an inflammatory reaction explained the acute symptoms in the other two. The varied symptoms in the remaining patients are summarized in Table I. The complaints that necessitated surgical operation in three of these patients undoubtedly were related to disease processes other than the ganglioneuroma.

Ganglioneuromas are not radiosensitive, and complete surgical ex-

tirpation is the accepted treatment. That incompletely removed ganglioneuromas, even those not found to contain undifferentiated cells, can recur and grow rapidly is illustrated in the case reported by Wyman and associates.[31]

Summary

Detailed pathologic studies have been made in a case of ganglioneuroma of the duodenum. This apparently is the first such lesion in this part of the intestine to be reported.

Search of the available literature revealed records of 11 ganglioneuromas of the gastrointestinal tract. Five of these tumors were in the appendix, two in the ileum, and two in the stomach; the remaining two involved the ileum, cecum, and ascending colon. One of the 12 tumors was clinically malignant, and one other had histologic features of malignancy.

Complete surgical extirpation is the accepted treatment.

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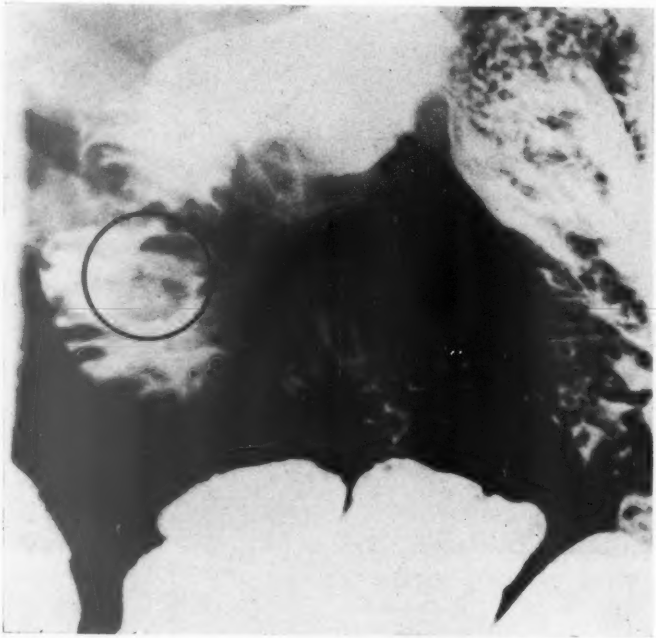
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Polypoid lesion in second part of duodenum, outlined by barium.

FIG. 2. Section of entire tumor, with small mucosal ulcer at upper right. Single cluster of Brunner's glands is seen within the tumor. Capsule is not shown at left because of oblique cut through tissue. Hematoxylin and eosin stain. × 12.



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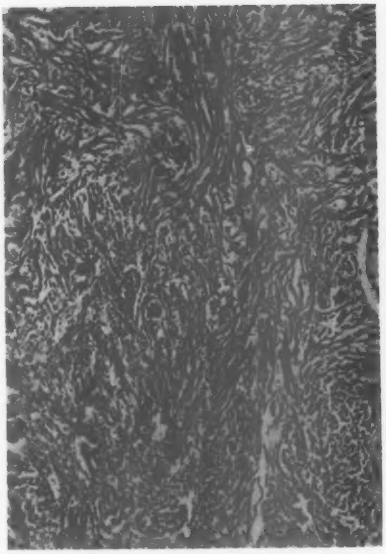

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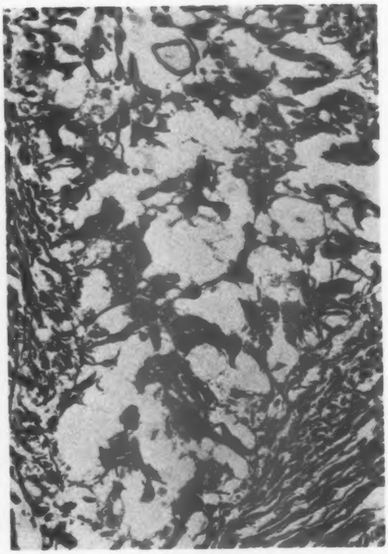

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FIG. 3. Ganglion cells sparsely scattered in predominantly neurofibrillar stroma. Bodian stain. × 75.

FIG. 4. Loose cluster of small ganglion cells. Bodian stain. × 200.

FIG. 5. Large, binucleate, multipolar ganglion cell and several smaller ganglion cells. Bodian stain. × 700.

FIG. 6. Large ganglion cell with multiple processes and a satellite. Bodian stain. × 700.

FIG. 7. Unusual gland-like structures within mucosa at left. Duplication and distortion of muscularis mucosae may be noted. Hematoxylin and eosin stain. × 40.

A CYST IN THE PRIMORDIUM OF THE TRICUSPID VALVE OF AN ABNORMAL HUMAN EMBRYO *

JAMES E. KINDRED, Ph.D.
From the Department of Anatomy, School of Medicine, University of Virginia, Charlottesville, Va.

Recently, during the examination of serial sections of a number of young human embryos, I found a cyst in the primordium of the tricuspid valve of an embryo whose crown-rump length was 10 mm. Anderson and Dmytryk[1] indicate that such a cyst is unique because of its occurrence in an embryo and its position in the embryo. It has been suggested that similar cysts, when found in the fully formed adult heart, have had an embryonic origin and that they could represent rests cut off from the entoderm of the nearby pharynx,[2,3] from the primitive epimyocardial mantle,[4,5] or from any neighboring epithelial membrane.[6]

The references to cysts of the epimyocardium cited by Davis[7] in his description of human cardiogenesis have not been mentioned in any of the recent papers nor has the later reference to such cysts by Heuser[8] been noted. In neither of these papers was there any description of the histologic character of the early epimyocardial mantle nor of the cysts. Hence in this brief paper the histologic characteristics of the cyst in contrast with those of the primitive epimyocardial mantle of a 5-somite embryo are presented.

REPORT OF CASE

The embryo in which the cyst occurred was obtained from a ruptured tubal pregnancy and when it was sketched at the time of sectioning, the body was observed to be twisted. There was a wide lumbosacral defect in the nervous system. Its menstrual age was 60 days, from which its length, according to Mall's formula,[9] should have been about 15 mm. rather than 10 mm. Upon sectioning, the discrepancy between the measured length and the length calculated from age was shown to be caused by twisting of the body and adhesions of the umbilical cord around the rump. The degree of organogenesis was that of a 15 mm. embryo, as can be seen from the stage of development of the organs shown in Figure 1.

The cyst stood out sharply in the endocardial tissue of the primordium of the tricuspid valve. Its topographic relations are shown

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in Figure 1, which is a photograph of a cross section of the body at the level of the pericardial cavity. The diameters of the cyst were 0.077 mm. long and 0.055 mm. wide. Since it extended through five sections at a thickness of 10 μ per section, its thickness was 0.05 mm. The diameters of the lumen were 0.02 mm. $\times$ 0.036 mm. The epithelium of the cyst (Figs. 2 and 3) was pseudostratified columnar and simple low columnar. There appeared to be a basal layer of columnar cells with large ovoid nuclei (sample measured 10 $\mu \times 4\ \mu$ in diameter) in which the chromatin varied depending upon the stage of activity. The nuclei were not homogeneous, but showed chromatin grouping which is characteristic of rapidly growing cells. In some cells the chromatin was in small granules on delicate linin strands with clear, wide areas of parachromatin in the background. In others, the chromatin was in coarse globules as if in the early prophase and the parachromatin had diminished. Strands of chromatin might cross the nucleus in the coarser types. The nucleoli were small and there might be several of them per nucleus. The nuclear membrane was dense but not thick. Where the cytoplasm was not vacuolated, it was lightly acidophilic and homogeneous. The basement membrane was very delicate and where seen it had been detached from the proximal ends of the basal cells. These had small phalangeal processes which were acidophilic in reaction. One cell was observed in mitosis. In that cell which was in the telophase, the chromatin was agglomerated at the poles of the spindle and the cytoplasm was dense, forming two sharply defined cones joined at the midpoint where the cell was about to divide. The cell membrane was very sharply defined and the cytoplasm was darkly acidophilic (Fig. 3).

There seemed to be an incomplete layer of surface cells lining the lumen. These were separated from the basal layer by a zone of vacuoles which lay in the distal ends of the basal cells. The nuclei in the cells of the surface layer were more vesicular than those of the basal cells, more irregular in shape, and the chromatin was more nearly uniform in distribution. Some of the nuclei had small lobules and most of them gave the impression of having been fixed while in motion. The nucleoli were small, and were usually present. The cytoplasm bordering the lumen was dense and in some places there was an acidophilic cuticle. In other cells the cytoplasm was vacuolated and stringy. The epithelium was not a well defined layer such as that which lines the esophagus and branchial pouches in the same region. Some degenerated cells had been sloughed into the lumen and there were some pyknotic nuclei in the cells of both layers.

External to the cyst there was an area of shrinkage between the basement membrane and the ensheathing cells of the delicate lamina propria. This was made up of a single layer of fibroblasts, circularly arranged parallel with the basement membrane. This layer alone separated the cyst from the endothelium of the lumen of the atrioventricular canal. On the other side of the cyst the fibroblasts merged with the endocardial tissue of the valve primordium. No vasa or nerves were observed. The cyst was quite independent of any surrounding epithelial structures (using the term epithelium in its broadest sense). Contrast of its nuclei with those of the nearest epithelial structures—the endothelium of the heart—showed that the nuclei of the endothelial cells were larger and plumper; the nuclear membrane was thinner; there was usually one large acidophilic nucleolus in the cells of the endothelium. Their nuclear chromatin was faintly stained; and there was a large amount of translucent bluish parachromatin between the chromatin strands. Consequently, it is believed that the cyst did not arise from differentiated endothelium.

Discussion

Examination of the descriptions of myocardial cysts and assessment of their presumed etiology, as given in some of the few papers which have appeared on this subject, revealed that there are three chief suggestions as to their origin: (a) They may arise as buds from the primitive cardiogenic plate at the time the heart is being molded[1,4,5]; (b) they may arise from entodermal diverticula which are cut off as the primitive mesocardium is formed below the foregut[2,3]; and (c) they may arise from any neighboring epithelium.[6] These suggestions are supported by topographic rather than histologic or embryologic evidence.

In examining the fundamental paper on human cardiogenesis by Davis,[7] I found mention of, and very brief reference to, the formation of cysts from the epimyocardial mantle (cardiogenic folds) at the time the epimyocardium was closing around the endothelial tubes ventral to the foregut in a 14-somite embryo. The same statement was made by Heuser[8] from the study of the heart of the same embryo. In neither of these papers was there any reference to the histologic characteristic of either the normal cardiogenic plates at this stage nor of the so-called cysts. These observations, however, do support topographically and embryologically the view of the etiology of the cysts from the epimyocardium.

Since we have in our collection a well preserved human embryo of 5

somites in which the heart is at the stage described by Davis[7] as about the time when myocardial cysts may form, I examined the cardiogenic folds or epimyocardial mantle (the visceral layer of the primitive pericardial cavity which gives rise to the myocardium) not only in regard to topography, but also histology. The topography of the area which has been studied is shown in Figure 4 and the histologic details of the epimyocardial mantle in Figure 5.

The cells of the cardiogenic plate in the area where it was folding to form the atrioventricular sulcus were columnar, and closely packed; the nuclei were ovoid; the chromatin was granular; the nucleoli were small; and the nuclear membrane was distinct. A few horizontally placed basal cells were present. The fold of the epimyocardium which was pushing in from the right side of the section could, by overgrowth into the subpharyngeal space, give rise to a cyst which, if cut off, would eventually lie in the valvular region of the heart. While the epithelium was not identical with that of the cyst, it was basically similar to it, and it is conceivable that the epithelium could change from the simple to the slightly pseudostratified type following proliferation within a closed space.

On the basis of this comparison, it is believed that the topographic and histologic evidence supports the view that the cyst described in this paper has arisen from an aberrant proliferation and sequestration of a portion of the primitive epimyocardial mantle at a stage comparable to that of the 5-somite embryo, at which stage there is a proliferation of the cardiogenic folds to form the atrioventricular sulci. Such cysts may be associated with general overgrowth of the epithelial tissues of the whole embryonic body and may rarely occur locally in normal development. In the embryo from which the cyst was obtained there was overgrowth of the neural epithelium and otic primordia, together with abnormal development of the caudal part of the body, including fusion of the umbilical cord with the rump and dislocation of the hind limbs.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Photomicrograph of the anterior surface of a transverse section (10 μ thick) through the thorax of the abnormal embryo to show the position of the cyst (C) in the primordium of the heart valve. The atrium (A) is congested and the lumina normally present in the bulbus cordis (B) have been obliterated. The tracheal primordium (T) is less well developed than normal. The neural tube (N) is in a condition of myeloschisis, and the ventral body wall is deficient below the septum transversum. Hematoxylin and eosin stain. × 18.

FIG. 2. Photomicrograph of primordium of valve showing the cyst, from the section shown in Figure 1. The irregular character of the epithelium may be noted. At about 9 o'clock, a cell in mitosis can be seen. The delicate membrane above the cyst is the endothelium of the valve. × 413.

FIG. 3. Photomicrograph of the cyst to show the arrangement of the cells at a higher magnification. A mitotic figure can be seen at about 9 o'clock. × 1,370.

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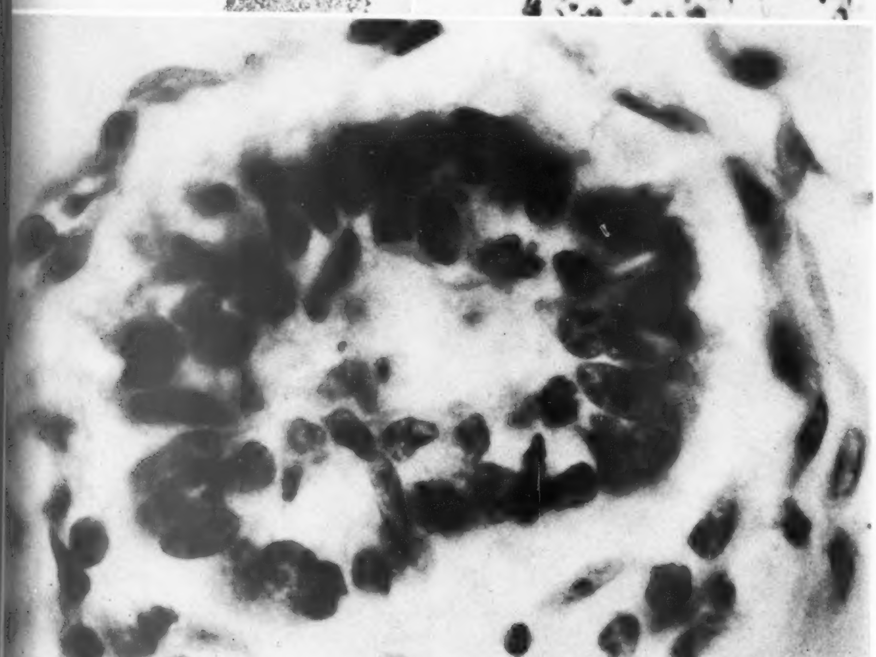

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FIG. 4. Photomicrograph of the anterior surface of a transverse section (10 μ thick) through the level of the primordium of the heart of a 5-somite human embryo. There is an open neural groove (N) below which lies the broad, thin-walled pharynx (P); and below this again is the myoendothelial space (M) occupied by the endocardial primordia (E). The thick-walled cardiogenic fold (F) at the right is the fold at the level of the molding of the left atrioventricular sulcus. Hematoxylin and eosin stain. × 120.

FIG. 5. Photomicrograph of the tip of the atrioventricular fold from the same section shown in Figure 4 to demonstrate the characteristics of the epithelium. Of note are the elongate cells, and the resemblance between this epithelium and that of the cyst. × 1,600.

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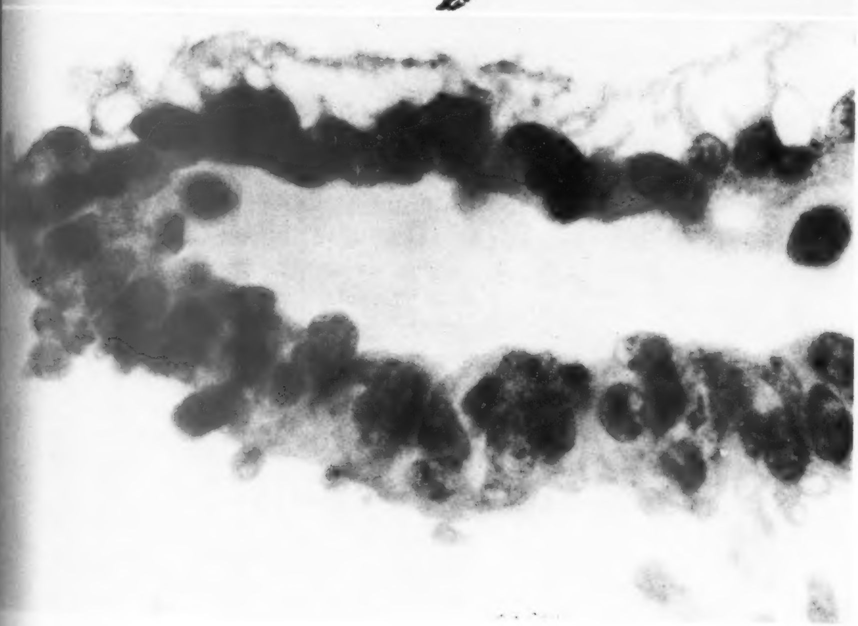

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STREPTOCOCCAL CARDIAC LESIONS IN RABBITS *

JOEL G. BRUNSON, M.D.; PETER E. FEHR, M.D.,† and RICHARD L. DAVIS, M.D.
From the Department of Pathology, University of Minnesota Medical School, Minneapolis 14, Minn.

The lesions produced in rabbits by carotid-jugular cross circulation or temporary carotid-jugular shunts have been described in detail in previous papers.[1,2] These studies have shown that fibrinoid lesions confined to the coronary arteries and cardiac valves can be produced in a high percentage of rabbits by either of these two methods when small amounts of heparin are used as the anticoagulant. It was shown also that the administration of other substances such as sodium polyanethol sulfonate (Liquoid), cortisone, meningococcal endotoxin, colloidal iron, or Thorotrast in conjunction with the shunting procedures resulted in the development of diffuse fibrinoid lesions which involved the lungs, heart, liver, spleen, and kidneys. When either colloidal iron or Thorotrast was used, it was observed that these substances were deposited within the areas of fibrinoid deposition, and admixed with this material beneath the endothelium of the coronary arteries and within the substance of the cardiac valves. Similar diffuse fibrinoid lesions were produced in rabbits given meningococcal endotoxin in conjunction with the administration of cortisone,[3] sodium polyanethol sulfonate,[4,5] colloidal iron,[6] or Thorotrast.[7] These observations supported the hypothesis that cross circulation or temporary arteriovenous shunt procedures acted in a manner similar to an intravenous injection of gram negative bacterial endotoxin. Thomas, Denny, and Floyd[8] reported that fibrinoid lesions of the coronary arteries and cardiac valves were produced in rabbits infected with group A hemolytic streptococci and given an intravenous injection of meningococcal endotoxin. Similar lesions were observed in rabbits given endotoxin after infection with pneumococci.[9]

As a further test of the similarity of action of cross circulation or temporary arteriovenous shunts to the action of intravenous endotoxin, it seemed reasonable to determine what lesions would be produced in rabbits by the administration of streptococci in conjunction with these procedures. In view of the demonstration that large particulate matter

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such as colloidal iron or Thorotrast could be localized beneath the endothelium of the coronary arteries and within the valves by these techniques,[2] it seemed logical to believe that microorganisms might be localized in similar sites, with the production of progressive cardiac lesions which could be studied over a longer period of time. This paper, therefore, deals with the production of such bacterial cardiac lesions in rabbits subjected to cross circulation or temporary arteriovenous shunt procedures.

Materials and Methods

A group of 114 hybrid albino rabbits of both sexes, weighing 1.0 to 1.5 kg., were used in the experiments. They were fed Purina rabbit pellets and had free access to water.

Carotid artery-jugular vein cross circulation or temporary shunts were performed with polyethylene tubing in a manner similar to that described in previous papers.[1,2,10,11] The animals were given 10 mg. of heparin sodium intravenously just prior to cannulation of the neck vessels, and circulation through the catheters was carried out for a maximum period of 30 minutes.

A strain of *Streptococcus viridans,* obtained from Dr. B. J. Clawson, was used in the experiments. Three ml. of a 24-hour broth culture were injected into the venous catheter or marginal ear vein of each of the animals during, or at various times prior to, or after cross circulation. The numbers of animals used in the various experiments and the time of administration of streptococci are indicated in the text.

The animals died or were sacrificed 6 days to 2 weeks following the administration of streptococci. Routine postmortem examinations were performed and the tissues were fixed in 10 per cent neutral formalin. Sections from the lungs, heart, liver, spleen, kidneys, and adrenal glands were examined routinely. A minimum of ten blocks from the heart of each animal was studied microscopically. Hematoxylin and eosin stains were used routinely, but many additional sections were stained with the periodic acid-Schiff method, the Gram stain for bacteria, and the Perls stain for iron.

Results

The numbers of animals used in the various experiments and the results of these procedures are summarized in Table I. None of the animals subjected to cross circulation alone developed bacterial cardiac lesions, and only two of 26 control animals given streptococci alone showed gross valvular vegetations. These two animals were among eight which were given streptococci and placed on their backs for 30 minutes following the injection. In contrast, 22 of 72 animals given

streptococci in conjunction with the cross circulation or temporary shunt procedures showed the presence of gross valvular lesions. In all instances the vegetations were confined to the mitral or aortic valves, or both, and no lesions of the tricuspid or pulmonary valves were observed grossly or microscopically. The gross vegetations were the only

TABLE I

Incidence of Cardiac Lesions Produced by Carotid-Jugular Cross Circulation or Temporary Carotid-Jugular Shunt when Performed in Conjunction with the Administration of Streptococci

First procedure	Interval	Second procedure	Death	Number of animals	Gross valvular vegetations	Microscopic cardiac lesions		
						Valvular	Myocardial	Fibrinoid
Cross circulation			15 days	16	0	6	10	3
Streptococci			8 days	10	0	0	4	2
Streptococci plus ligation of carotid-jugular veins			8 days	8	0	5	6	2
Streptococci (on back 30 minutes)			8 days	8	2	4	1	3
Cross circulation	Simultaneous	Streptococci	8 days	11	5*	8	11	5
Cross circulation	12 hours	Streptococci	6 days	15	4	12	15	10
Cross circulation	18 hours	Streptococci	8 days	14	4	12	14	12
Streptococci	18 hours	Cross circulation	9 days	16	4	11	11	7
Arteriovenous shunt	18 hours	Streptococci	15 days	8	3	8	5	5
Streptococci	18 hours	Arteriovenous shunt	16 days	8	2	7	6	6

* Mitral and aortic valves involved in each animal.

valvular lesions in which bacteria were demonstrated microscopically. It is of some interest that there was a similar incidence of valvular vegetations in animals subjected to cross circulation or arteriovenous shunt either 18 hours prior to or after the administration of streptococci.

The gross valvular lesions varied somewhat in appearance and severity. The vegetations were usually firm, non-friable, opaque, shiny, nodular elevations which tended to be confluent and were located along

the free margins of the aortic cusps or mitral leaflets. In many instances the valves were markedly distorted (Fig. 1) and the left ventricle was dilated. In other animals the vegetations were less numerous and only scattered, discrete, small, firm nodules, 1 to 2 mm. in diameter, were observed. These were present occasionally along the chordae tendinae and the endocardial surface of the left ventricle below the mitral valve. Less commonly, the vegetations were shaggy and friable. In one group of animals, as noted in Table I, both the aortic and mitral valves were involved. The vegetations in these cases were quite large and were present on both surfaces of the valves. In addition to left ventricular dilatation, extensive areas of myocardial hemorrhage and necrosis were observed frequently.

Microscopically, a variety of changes was noted. In each of the sections taken from the gross valvular lesions, clumps of bacteria were observed, but none was demonstrated in sections from other valves. These bacterial vegetations were associated with extensive deposits of fibrinoid and there was a marked cellular reaction which often extended throughout the substance of the valve. The cellular response consisted chiefly of large mononuclear cells with basophilic cytoplasm and indistinct borders, many Anitschkow cells, occasional clumps of heterophils,* and large numbers of spindle-shaped cells resembling fibroblasts. In many instances the vegetation appeared partially or completely covered by endothelium. Masses of calcium were observed occasionally within the vegetations (Figs. 2 and 3). The bacterial lesions were particularly severe in the animals given streptococci simultaneously with cross circulation. In these animals, it was difficult to recognize the valvular structure with certainty, as it appeared to be incorporated into the vegetation.

In addition to these valvular changes, other lesions were present which had not been detected grossly. These consisted of small nodular elevations along the surfaces of the valves, composed of clumps of fibrinoid material, with varying numbers of mononuclear and Anitschkow cells, and some fibroblasts. These nodules were covered by endothelium, or lay beneath endothelium continuous with that of the valve (Fig. 4).

The myocardial alterations, likewise, were both bland and septic. In many hearts small areas of acute necrosis, with numerous heterophilic and mononuclear cells, were present. In several of these areas

* According to Maximow and Bloom, the heterophilic cells of rabbits correspond to polymorphonuclear neutrophils of man, and are characterized by granules which usually stain with acid dyes. They are sometimes referred to as pseudo-eosinophils. (Maximow, A. A., and Bloom, W. A Textbook of Histology. W. B. Saunders Co., Philadelphia, 1948, ed. 5, p. 50.)

it was possible to demonstrate clumps of organisms by the Gram stain. In other hearts there were extensive areas of ischemic necrosis of the muscle which were associated with arterial occlusion by hyaline fibrinoid material (Fig. 5). These intraluminal accumulations of fibrinoid occasionally contained streptococci and, more rarely, small clumps of calcium salts. In these instances they appeared to represent emboli from the valvular vegetations. In one case it was possible by serial sections to trace such an embolus from the root of the aorta into one of the major coronary arteries. In most instances, however, this fibrinoid material appeared bland, and was associated with the presence of similar material beneath the arterial endothelium. These lesions were quite numerous, and many of the intramural and pericardial coronary arteries showed the presence of extensive subendothelial and perivascular accumulations of fibrinoid with a varying degree of cellular reaction. The cells were composed almost entirely of Anitschkow cells in some instances, and in other instances appeared to be composed largely of heterophils (Figs. 6 to 10).

In the animals subjected to cross circulation alone, only three showed the presence of coronary or arterial fibrinoid lesions. In many, however, the valves appeared thicker than normal and there were numerous large mononuclear cells. Occasional valves showed the presence of pigment-containing macrophages which gave positive reactions for iron by the Perls method. Myocardial changes were observed in 10 of the 16 animals subjected to this procedure. These consisted of resolving areas of necrosis of muscle with many capillaries, loose fibrous tissue, and varying numbers of mononuclear cells.

No other consistent lesions were observed in these animals although, occasionally, pulmonary edema and chronic passive congestion of the liver were present. In several of the animals, branches of the pulmonary arteries were occluded by thrombi or emboli which contained clumps of bacteria and calcium salts (Fig. 11). Since no lesions of the right side of the heart were observed, it is possible that these may have originated within the arteries, in a manner similar to that observed in previous experiments in which colloidal iron or Thorotrast was deposited in the walls of the pulmonary arteries.[2] In only an occasional animal were renal or splenic infarcts observed.

Discussion

Lillehei and coworkers[11] reported the spontaneous development of bacterial endocarditis in dogs in which permanent arteriovenous fistulas of the iliac or femoral vessels had been created. These cardiac lesions occurred in approximately 50 per cent of the animals in which shunts

had been present for 4 weeks or longer. In other experiments[12] it was demonstrated that all dogs in which such fistulas were present developed bacterial endocarditis after multiple intravenous injections of bacteria.

In the present experiments it has been shown that rabbits develop bacterial cardiac lesions following intravenous injection of *Strep. viridans* when these organisms are given in conjunction with carotid-jugular cross circulation or temporary carotid-jugular shunt. The size, location, and duration of the shunt, as well as the number of injections of bacteria and the time required for the development of the lesions, differ from those in Lillehei's[11,12] experiments. The incidence of gross valvular involvement is distinctly less than that observed in dogs, but the high incidence of microscopic cardiac alterations in rabbits suggests that certain similar mechanisms may be involved in the pathogenesis of the lesions in both groups of animals.

In previous experiments it has been shown that cardiac fibrinoid lesions in association with muscle necrosis and cellular reaction, and valvular hemorrhages and cellularity, are regularly present in rabbits after cross circulation or temporary shunt procedures.[1] The results of the present experiments indicate that such fibrinoid lesions may regress within a period of 2 weeks following the shunt procedures unless some additional stimulus is provided. Although bacteria were not demonstrated in a majority of the cardiac lesions in the present experiments, it seems logical to believe that they were associated with the persistence and progressive development of the lesions. In previous experiments it was observed that granules of iron or Thorotrast were present within the cytoplasm of vascular endothelial cells when these substances were given in conjunction with the carotid-jugular shunting procedures.[2] These observations suggest that these cells may have or may develop phagocytic properties, and that bacteria may also be ingested when they are administered in association with the temporary shunt techniques. It is possible that they were present in such small numbers as not to be seen in histologic sections, or that they had been present and had disappeared during the long interval from injection of the bacteria to sacrifice of the animals. It is also possible that, during their destruction or degradation, certain products might have been formed or released which contributed to the progressive nature of the lesions. These experimental observations suggest, therefore, that cross circulation or temporary arteriovenous shunt may "prepare" the heart for secondary bacterial invasion, and that this preparation is associated with structural or functional alterations in the vascular endothelium coupled with the production and deposition of fibrinoid.

The equal incidence of cardiac lesions in rabbits given streptococci before or after cross circulation or temporary shunt does not invalidate this theory. It has been shown by Thomas *et al.*[13] that rabbits given intravenous streptococci may have a bacteremia for several days and, even after blood cultures become sterile, the same strain of organisms may be recovered from cultures of such organs as the heart, kidney, or liver. In subsequent experiments it was shown that after blood cultures of streptococci-infected rabbits had become sterile, the administration of cortisone was associated with the reappearance of these organisms into the blood stream.[13] It seems reasonable, from these data, to believe that the animals given streptococci 18 hours prior to carotid-jugular shunt procedures either had a bacteremia at the time of these procedures, or that such procedures may be associated with a "reactivation" of dormant or sequestered organisms which then localize in certain altered foci in the heart.

It is also probable that an occasional animal is spontaneously "prepared" so that enormous numbers of bacteria, such as used in the present study, may bring about the development of cardiac fibrinoid-bacterial lesions. The fact that small numbers of rabbits in each of the groups given streptococci alone developed such lesions is evidence that this may be true. Other evidence that a state of preparation may exist prior to any experimental procedures is seen in the reaction of rabbits to intravenous Gram-negative endotoxin. In an occasional animal, a single such injection is followed by the development of diffuse fibrinoid lesions,[14] but in a majority of rabbits two properly spaced injections of endotoxin are required for the development of such lesions.[15]

Although the mechanisms involved in these experimental procedures are not entirely clear at the present time, the results suggest that carotid-jugular cross circulation or temporary carotid-jugular shunt procedures may allow localization of specific microorganisms in the valves and arteries of the heart. Such localization appears to depend upon some change in the integrity of the vascular endothelium associated with the deposition of fibrinoid. These experimental procedures, it is suggested, may permit closer investigation of the rôle of group A hemolytic streptococci in the pathogenesis of rheumatic carditis.

Summary

Hybrid albino rabbits were subjected to carotid-jugular cross circulation or arteriovenous shunts for maximum periods of 30 minutes. A single intravenous injection of *Streptococcus viridans* was given during, or at varying times prior to or after, the shunting procedure. Approximately 30 per cent of the animals developed gross bacterial lesions of

the mitral or aortic valves, or both, but microscopic cardiac changes were observed in approximately 90 per cent. These consisted of extensive areas of myocardial necrosis and accumulations of mononuclear, heterophilic, and Anitschkow cells about the areas of necrosis and within the valves. In association with these lesions extensive deposits of fibrinoid material were present in the valves, walls of the coronary arteries, and perivascular spaces. No consistent changes were observed in other organs. These data indicate that specific microorganisms may be localized to the heart by means of the shunting procedures, and suggest that such localization is associated with structural or functional alterations in vascular endothelium together with the production and deposition of fibrinoid material.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

All sections were stained with hematoxylin and eosin.

FIG. 1. Gross photograph of the heart of a rabbit subjected to the cross circulation procedure and given a single intravenous injection of *Streptococcus viridans* 18 hours later. The animal died 6 days following the injection of bacteria. The left ventricle has been opened to show the marked distortion of the mitral valve associated with the presence of large, firm, nodular vegetations which involve both portions of the valve. × 3.

FIG. 2. Section from the aortic valve of a rabbit given streptococci simultaneously with cross circulation. The animal died 8 days following the procedure. An extremely large bacterial vegetation involves the entire thickness of the valve, and numerous large clumps of bacteria are present. There is also an extensive inflammatory reaction extending into the root of the aorta. × 12.

FIG. 3. Mitral valve from the same animal as that from which Figure 2 was taken, showing a similar bacterial vegetation which in this instance appears largely localized to the atrial surface of the leaflet. × 12.

1

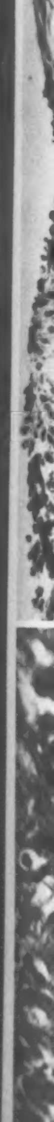
3

FIG. 4. Section from the mitral valve of a rabbit given streptococci 18 hours after carotid-jugular cross circulation. A nodular accumulation of fibrinoid material is present just beneath the endothelium of the valve, with a minimal cellular reaction. The animal was sacrificed 8 days after the administration of bacteria. × 250.

FIG. 5. Section from the myocardium of an animal given streptococci simultaneously with the shunting procedure. There is an extensive area of necrosis of muscle about an intramural coronary artery with hemorrhage and cellular reaction which consists of Anitschkow, heterophilic, and mononuclear cells. Most of the arterial wall is replaced by fibrinoid material and bacteria were demonstrated in the wall of the vessel by the Gram stain. The animal was sacrificed 8 days after the shunt procedure. × 250.

FIG. 6. Section from the myocardium of a rabbit given streptococci 18 hours prior to the arteriovenous shunting procedure and sacrificed 16 days following the shunt. An extensive deposit of fibrinoid material surrounds one end of a small intramural coronary artery, and extends out into the perivascular space. Moderate numbers of Anitschkow and mononuclear cells are present. × 250.

FIG. 7. Similar myocardial lesion from another animal given streptococci 18 hours after the shunting procedure. There is more extensive fibrinoid deposition in the wall of the vessel and a somewhat more pronounced cellular reaction. The animal was sacrificed 15 days after the temporary arteriovenous shunt. × 250.



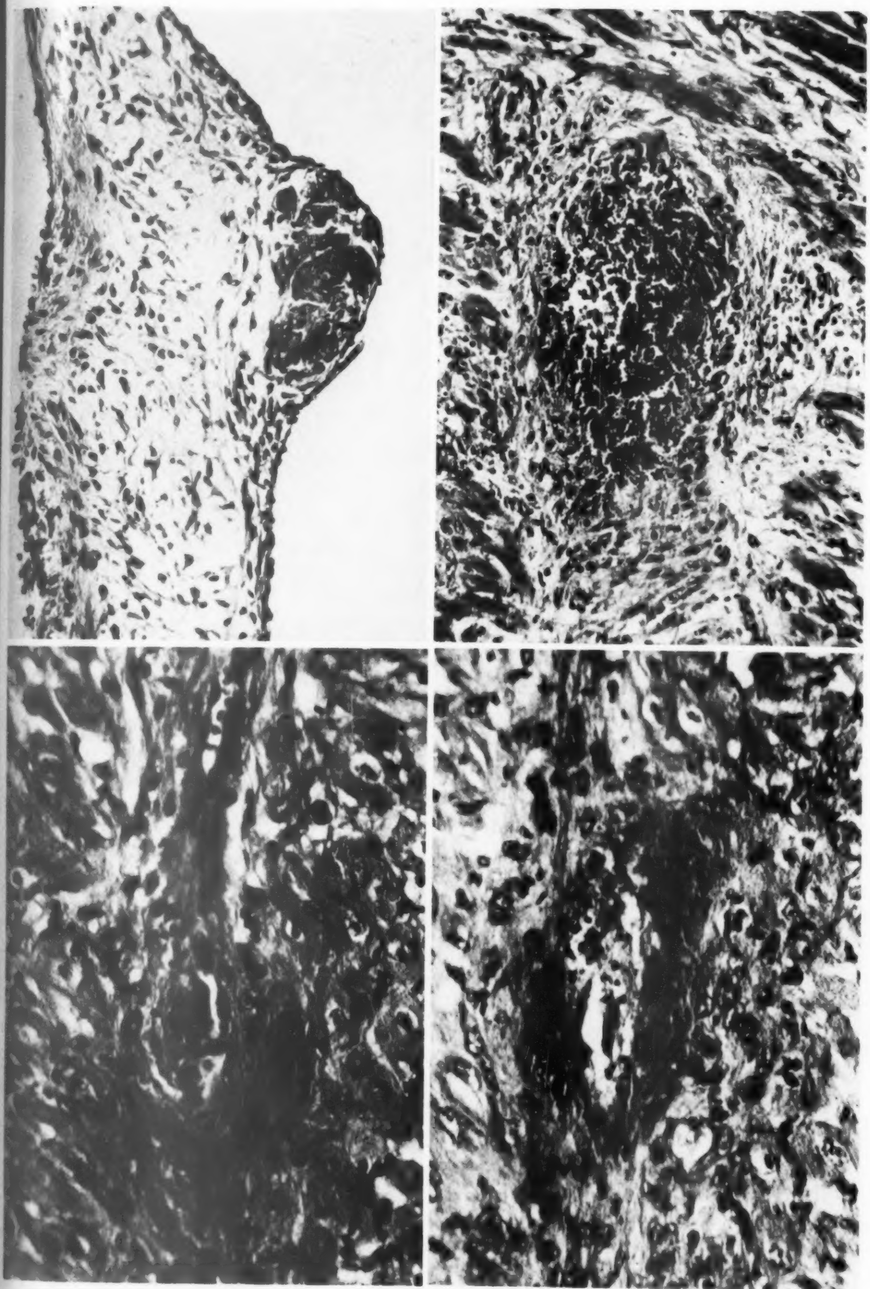


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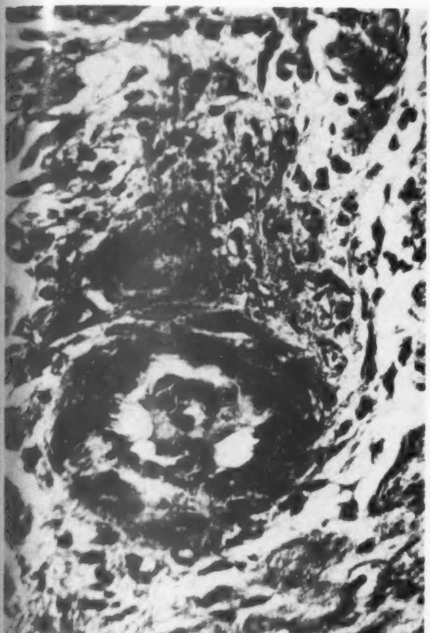
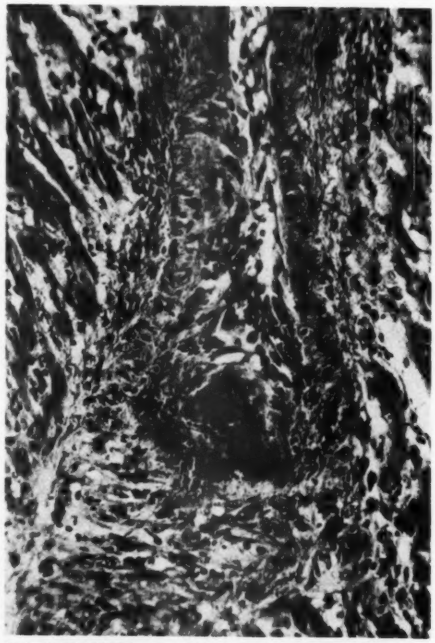
FIG. 8. Pericardial coronary artery from a rabbit subjected to cross circulation and given streptococci 18 hours later. The animal was sacrificed 15 days after the administration of bacteria. There is almost complete replacement of the arterial wall by fibrinoid, and some of this material is also perivascular and associated with a mononuclear cellular reaction. × 350.

FIG. 9. Section from the myocardium of a rabbit given streptococci 18 hours after cross circulation and sacrificed in 8 days. There is a large deposit of fibrinoid material in the wall of an intramural coronary artery which projects into the lumen. There also appears to be extensive endothelial proliferation and a heavy perivascular cellular reaction. × 250.

FIG. 10. Higher magnification of the cellular reaction shown in Figure 9. Numerous Anitschkow and mononuclear cells surround the hyaline fibrinoid material. × 450.

FIG. 11. Section from the lung showing a large pulmonary artery which is occluded by a thrombotic mass which contains large clumps of calcium and micro-organisms. There is evident organization and recanalization of the thrombus. The rabbit was given streptococci 18 hours prior to arteriovenous shunt and sacrificed in 8 days. × 60.





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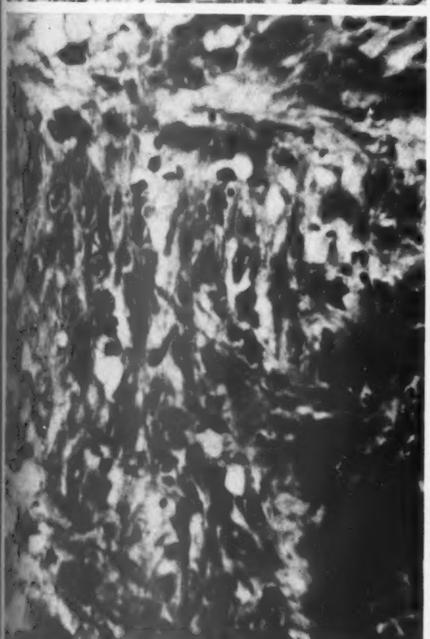

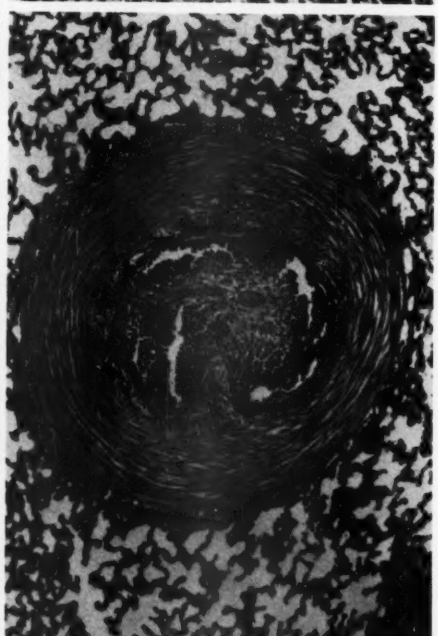

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ACUTE GLOMERULONEPHRITIS

A Histopathologic Study by Means of Thin Sections *

Edith Grishman, M.D., and Jacob Churg, M.D.

From the Laboratories, Division of Pathology, the Mount Sinai Hospital, New York, N.Y.

The classical concept of acute glomerulonephritis is that of an inflammation of glomerular capillaries accompanied by a striking overgrowth of endothelial cells. This interpretation (Herxheimer,[1] Fahr,[2] Bell[3]) has been thoroughly accepted for the past 30 years, although previously the changes in the glomeruli had been ascribed either to epithelial hyperplasia (Hansemann[4]), or, before that, to connective tissue proliferation (Klebs[5]). In recent years, McManus[6] called attention to the alteration of the intercapillary area in which he found edema and exudation of leukocytes. Lately, the original concept of Klebs has been revived by Jones[7,8] who postulated that the proliferating cells of acute glomerulonephritis are the connective tissue cells or histiocytes in the glomerular mesangium, and not endothelial cells.

The work of Jones[7,8] was based essentially on the use of thin sections stained by newer histologic methods (periodic acid-Schiff's reagent, periodic acid-silver methenamine). Thin sections, in general, are very helpful in the study of glomerular structure.[9] With conventional microtomes and paraffin embedding, 2 μ is about the lowest practical limit of thickness. Even that thickness is often excessive for detailed analysis of glomerular inflammation. With the advent of ultramicrotomes, designed primarily for the needs of electron microscopists, and special embedding methods, it is now possible to obtain serial sections at a thickness of 0.5 μ or less, and sufficiently large to contain a number (up to 30) of glomeruli. Application of this technique to the study of glomerulonephritis has been reported upon briefly.[10] In the present communication, analysis of changes in acute glomerulonephritis will be given.

Material and Methods

Twenty-one consecutive cases of acute glomerulonephritis were selected from the files of the Department of Pathology of the Mount

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Sinai Hospital. Paraffin blocks were melted down and small pieces of tissue measuring 2 by 2 mm. were double-embedded in celloidin and paraffin.[11] Serial sections were cut on the Gettner-Ornstein ultramicrotome[12,13] at a thickness of 0.5 μ. Consecutive sections were stained with hematoxylin and eosin, periodic acid-Schiff's (PAS) reagent,[14] and a modification of Mallory-Roque aniline blue (chromotrope-aniline blue[15]). To obtain sufficiently intense coloration of the thin sections, the time in the staining solution had to be prolonged five to tenfold over that recommended for sections of standard thickness. In some cases, one of the sections of a series was also studied under the phase microscope.

Results

The histologic changes in glomeruli of acute glomerulonephritis, as seen in conventional sections, are well known and consist mainly of increase in size and cellularity of the glomeruli and ischemia of the capillaries. These changes take place in the three component spaces of the glomerulus: the capillaries, the intercapillary space (mesangium), and the epithelium-lined Bowman's space. They follow a certain pattern of development—first progression and then regression.

In the early cases the changes within the capillaries were characterized by swelling, focal proliferation, and occasional desquamation of endothelial cells and the accumulation of polymorphonuclear leukocytes in the lumina. The number of red blood cells usually was decreased. The earliest change of the mesangium consisted of edema which caused widening of the intercapillary space (Fig. 7). However, the lobular architecture of the glomerulus was well preserved at this stage, and the normal "cloverleaf" arrangement of the capillaries (Fig. 6) was easily recognizable.

In more advanced and more severe cases the changes of the mesangium became very prominent and were chiefly responsible for the typical picture of acute glomerulonephritis.

As the disease progressed there was further widening of the intercapillary space, which became infiltrated by cells and distended by edema fluid (Fig. 8). The cells were predominantly mononuclear, with scanty cytoplasm and nuclei similar to those of endothelial cells. Polymorphonuclear leukocytes were found also in the intercapillary space (Fig. 8).

The enlargement of the intercapillary space led gradually to a change in the appearance of the glomerular lobes which has been called clubbing or ballooning (Figs. 8 and 9). Instead of the usual shape of a grape cluster on a thin stalk, the lobule now became a ballooned glob-

ular structure occupying a large part of Bowman's space. The capillaries were spread apart, forming a wreath around the widened mesangium. Often they were delimited from the mesangium by a delicate endothelial basement membrane (Figs. 8 and 10). However, as the mesangium increased in size, the capillaries became more and more compressed. Sometimes they were partly detached from the surface membrane and surrounded by their own delicate membrane, as cells and edema fluid infiltrated the space between the two basement membranes (Fig. 11).

At its maximum, the ballooning of the lobule became so severe that it was difficult to distinguish the mesangium from the capillary lumina (Fig. 9). The structure of the lobule became markedly disorganized. The core of such a lobule consisted of numerous cells, mainly mononuclear, and fragments of fibers or membranes. The capillaries were either compressed or their limiting membrane was broken up into small pieces and no longer recognizable as a continuous outline. However, the epithelial basement membrane surrounding the lobule remained largely intact. The peak change was observed in cases of 10 to 20 days' duration. After about 3 to 4 weeks, mesangial edema was less severe and the capillary outlines were again visible. At that time, laying down of fibers was observed in the mesangium (Fig. 12), though some increase in fibers was seen as early as 11 days after the onset of illness.

The three cases showing the most prominent mesangial fibers had a history of from 3 weeks to 3 months representing a transition to the subacute stage of glomerulonephritis. The fibers stained bright red with PAS and blue with aniline blue stain. They were branching and tortuous, at first very thin but gradually increasing in thickness. As the fibers of the mesangium multiplied, the number of cells in this space became smaller and the size of the mesangium and of the lobules decreased. The capillary lumina again appeared patent, although they contained very few red blood cells (Fig. 12).

The epithelial cells usually showed only minor changes consisting of swelling and proliferation. Small crescents were occasionally observed, with deposition of delicate membranes between the cells. The visceral and parietal basement membranes usually remained normal in size and delicate in appearance. At the hilus there was often some mononuclear and polymorphonuclear leukocytic infiltration and edema occupying the space around the afferent and efferent arterioles. A varying degree of cellular infiltration and edema was found also in the periglomerular and intertubular stroma paralleling somewhat the involvement of the glomerular mesangium.

Table I summarizes the most important clinical and pathologic data

TABLE I

Clinical and Pathologic Data of Cases of Acute Glomerulonephritis

Case no.	Sex	Age	Duration of illness	Albuminuria	Blood pressure	Edema	Azotemia (blood urea nitrogen)	Cause of death	Glomerular histology: Capillaries	Mesangial edema	Leukocytes	Mononuclear cells	Fibers
		Yrs.			*mm. Hg*		*mg./100 ml.*						
1	F	22	?	?	?	o	?	Acute bacterial endocarditis	N	+	++	+	o
2	F	14	1 wk.	+++	130/105	++	?	Pulmonary edema	C	++	++	+++	o
3	M	25	?	±	114/60	o	?	Subacute bacterial endocarditis	C	++	++	++	o
4	M	8	?	++++	98/55	+	?	Staphylococcic sepsis	N	++	++	++	o
5	F	12	11 days	+	?	±	69	Purulent peritonitis	N	+	+	+	o
6	F	70	10 days	+++	180/90	+	83	Arteriosclerotic heart disease	N	+	++	++	++(A)
7	F	33	7 wks.	+++	140/94	o	N	Septic thrombophlebitis	N	+	++	+	±
8	F	32	3 wks.	++	110/82	+	130	Uremia	C	+++	+++	++	±
9	M	40	3 wks.	+++	170/50	+	230	Uremia	C	+++	++	+++	++
10	F	18	10 days	++	130/98	o	148	Subacute bacterial endocarditis, uremia	C	+++	++	+++	o
11	M	49	3 mos.	++	150/50	+	69	Lobar pneumonia	N	±	+	++	+++
12	M	18	2 wks.	+++	192/110	++	N	Pulmonary edema	Sl.C	++	+++	++	+
13	M	46	12 days	+++	200/110	++	110	Uremia, pulmonary edema	C	+++	++	+++	±
14	M	47	2 wks.	?	180/100	+	?	Lobar pneumonia	C	+++	+++	+++	o
15	M	52	5 wks.	++	270/?	++	35	Coronary heart disease	Sl.C	+	++	++	+(A)
16	F	12	5 wks.	?	154/110	++	?	Convulsions	Sl.C	+	++	++	++
17	F	3	11 days	±	140/100	++	N	Pulmonary edema	Sl.C	++	+	+++	+
18	M	50	2 wks.	++++	150/90	++	90	Uremia, pulmonary edema	C	+++	+++	++	+
19	F	60	1 wk.	+++	170/104	+	N	Diabetes, arteriosclerotic heart disease	N	+	++	+	±(A)
20	M	16	1 wk.	+++	170/100	++	N	Pulmonary edema	C	++	+++	++	o
21	M	21	6 days	±	150/108	++	66	Pneumonia	C	+++	++	++	o

N = normal, C = compressed, Sl.C = slightly compressed, A = arteriosclerotic change, ± to ++++ = degree of change, ± minimum and ++++ maximum.

of the cases studied. Although no consistent relationship existed between the clinical data and the histologic appearance of the glomeruli, there were two points of correlation worthy of note. In most of the examples characterized by the most severe changes of the mesangium, particularly edema and cell proliferation, the patients died in a state of azotemia. These cases showed the most marked lobular ballooning and incomplete separation of capillary lumina from mesangium. On the other hand, patients dying in nephritic pulmonary edema did not show such severe mesangial ballooning. Such cases were considered by Bell[8] to be of shorter duration. The patients apparently do not live long enough to develop uremia.

Discussion

Klebs[5] was the first to observe the increased cellularity of glomeruli in acute glomerulonephritis of scarlet fever. For his studies he used teased preparations of glomeruli which had dropped off the cut surface of kidney tissue. He concluded that the cellularity was due to an increase of cells of the interstitial tissue of the glomerulus causing compression of the capillaries. Since then the interpretation of the nature of these cells and of the primary lesion of acute glomerulonephritis has run a full circle, until recently Jones[7,8] proposed a concept similar to that of Klebs.

In general, our own findings agree with those of Jones.[7,8] There is no doubt that the mesangium plays an important rôle in acute inflammation of the glomerulus, and that compression and ischemia of the capillaries are caused by edema and cellular infiltration of the mesangium.

The existence of the intercapillary space and mesangium is still doubted by some authors (Allen,[16] Hall,[17] Mueller *et al.*[18]) while others, including several electron microscopists (Policard *et al.*,[19] Yamada[20]), have accepted the presence of intercapillary mesangium. Mesangial structures can be demonstrated in thin sections stained with PAS (Fig. 5). The normal intercapillary space contains a small number of cells and fibers. The latter are most numerous near the hilus, but can be found also in the centers of peripheral lobules. The lobules consist of capillaries arranged around a central mesangial stalk. In cross section such a lobule may resemble a clover leaf (Fig 6).

The cells occupying the mesangium of the normal glomerulus have been interpreted in various ways. Jones[7] considered them to be of connective tissue origin. Yamada[20] suggested that they may be similar to smooth muscle cells because of intracellular myofilament-like threads which he demonstrated with the electron microscope. The demonstration of intercapillary fibers was accomplished by Bensley

and Bensley[21] with the aid of a silver impregnation method. They concluded that they were dealing with reticulum fibers. Recent studies with the electron microscope have led Yamada to conclude that the fibrillar component of the mesangium is part of the framework of the basement membrane. Electron microscopy of the normal glomeruli has not yet revealed any fibers with the periodicity characteristic of collagen.

In acute glomerulonephritis there is marked increase of mesangial cells. It is debatable whether these cells are histiocytes which have migrated to the mesangium, or arise through proliferation of cells originally present in the mesangium. The fibers laid down in the mesangium in acute glomerulonephritis are variously interpreted as connective tissue fibers (Jones[8]), as reticulum fibers developing in relation to endothelial cells (McManus[6]), or as fibers arising through splitting of the inner layer of the capillary basement membrane (Bell[3]). Staining reactions are equivocal in this respect. The normal as well as the newly deposited fibers of acute glomerulonephritis stain blue with aniline blue and black with silver impregnation methods. They also give a bright red reaction with PAS which is unlike the reticulum fibers of the renal stroma. The latter reaction is that usually given by basement membranes. Yet in the combined colloidal-iron PAS stain, the two elements—basement membranes and normal intercapillary fibrils—can be stained differentially.[22] Perhaps the answer as to the nature of these fibers will be supplied by electron microscopy.

The structure of the capillary wall has been studied recently by several electron microscopists (Mueller *et al.*,[18] Yamada,[20] Hall,[23] Pease[24]). This wall consists of epithelial cells with foot-like processes ("pedicels," Hall) which rest upon the basement membrane. The capillary lumina are lined by markedly attenuated, perforated cytoplasm of endothelial cells. The basement membrane proper consists of three layers: the dense middle layer and the outer and inner layers of lower electron density. Hall[17] compared the width of the basement membrane in electron micrographs and in stained preparations under the light microscope. He suggested that the latter probably include, in addition to the basement membrane proper, the layer of endothelial cytoplasm and a layer of foot processes of epithelial cells.

Studies of abnormal glomeruli (glomerulonephritis,[7,25] hyaline deposits[9]) suggested the existence not of one but of two basement membranes, one corresponding to the epithelium and the other to the endothelium. Jones[7] believes that the endothelial and epithelial

basement membranes lie closely together around the capillaries, separated by an interstitial space which is usually too thin to be visualized. In the opinion of Bohle and Krecke,[25] the basement membranes normally fuse and appear as a single structure under the light microscope. According to them, the capillary or subendothelial basement membrane is a continuation of the subendothelial membrane of the afferent arteriole, whereas the subepithelial basement membrane is continuous with the basement membrane of Bowman's capsule and the proximal convoluted tubule. In the normal glomerulus the "endothelial basement membrane" can be seen only in the narrow part of the circumference of the capillary directed toward the center of the lobule. It appears as a delicate line separating the capillary lumen from the intercapillary space.

Jones[7] has stressed the rôle played by what he called the pericapillary space between the two basement membranes in the development of acute glomerulonephritis. He believes that edema fluid may strip the two membranes apart so that the capillaries may float relatively free in edema fluid. Similar observations were made by Bohle and Krecke,[25] who stated that under abnormal conditions, such as malignant nephrosclerosis or glomerulonephritis, the two membranes may separate from each other.

As mentioned before, electron microscopists believe that there is only one basement membrane. Their statements are based on observations of normal glomeruli. There is no question that under favorable conditions and particularly in abnormal glomeruli one can observe a thin membrane separating the capillary lumen from the mesangium (Fig. 8). Our observations also indicate that in inflammation and edema there is progressive separation of the capillary from the epithelial basement membrane and that the capillary lumen is surrounded by a delicate "endothelial basement membrane" (Figs. 7 and 11). It is difficult to interpret the exact nature of this membrane. It may represent endothelial cytoplasm stripped off the basement membrane, or it may include part of the basement membrane proper. Perhaps this point will also be settled in the future by electron microscopy. At some point the capillaries usually remain attached to the epithelial basement membrane. It is believed that the separation progresses from the mesangial stalk toward the periphery through gradual infiltration of the "pericapillary space." This confirms a similar observation of Bohle and Krecke.[25]

In severe cases of glomerulonephritis with conspicuous widening of the mesangium, the "endothelial basement membrane" frequently

was found to be broken up into several fragments instead of appearing as a continuous outline. It then became very difficult to interpret these fibrils as capillary basement membranes. However, by analogy to many places which still showed continuous outlines of capillary lumina, it was concluded that these fragments represented remnants of an "endothelial membrane." Bohle and Krecke[25] also observed that the capillary basement membrane in subacute glomerulonephritis rarely surrounds the entire capillary. They ascribed this phenomenon to the fact, however, that the membrane is a very delicate structure and therefore difficult to visualize. In agreement with other authors (Jones,[7,8] Bohle and Krecke[25]) we usually found continuity between the mesangial and pericapillary spaces.

A few words may be said about the classification of acute glomerulonephritis. It is usually divided into exudative and proliferative types. There are also rarer varieties such as acute membranous and hemorrhagic types. These latter are not represented in our material, and so will not be discussed. The exudative type is supposed to be characterized by the predominance of polymorphonuclear leukocytes, and the proliferative by the predominance of mononuclear cells. A glance at Table I shows the presence of cells of both types in every case, though the proportion varies. Early cases tend to have more leukocytes and late cases more mononuclear cells, though this relation is not constant. Except for true glomerular abscesses, most cases are of the mixed, proliferative and exudative type.

Summary and Conclusions

Twenty-one cases of acute glomerulonephritis were studied by means of thin (0.5 μ) sections.

The basic alteration in acute glomerulonephritis is inflammation of the intercapillary mesangium. The inflammation progresses through the stages of exudation, cellular proliferation, and resolution. The initial accumulation of leukocytes in the capillary lumina with swelling and some proliferation of endothelial cells is rapidly followed by edema, exudation, and proliferation of mononuclear cells in the intercapillary area. These intercapillary changes are mainly responsible for the capillary ischemia. In the later stages of inflammation, fibers may appear in the mesangium. This constitutes transition to subacute glomerulonephritis.

The nature of the mononuclear cells and of the fibers in the mesangium, and the changes in the capillary wall are discussed.

Except for true glomerular abscesses, the so-called exudative and

proliferative forms of acute glomerulonephritis merely represent two facets of the same disease process.

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LEGENDS FOR FIGURES

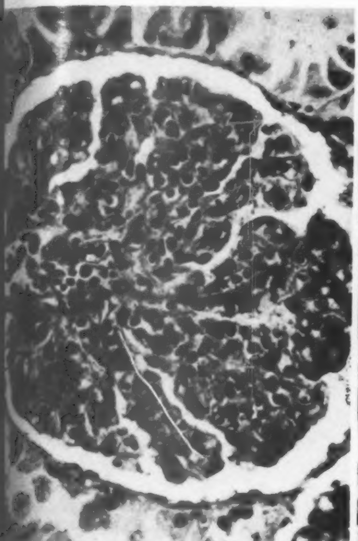
Figs. 1 to 4. Progressive stages of acute glomerulonephritis. Sections were cut at a thickness of 5 μ. Hematoxylin and eosin stain. $\times$ 300.

FIG. 1. Case 1, early stage. Polymorphonuclear leukocytes in capillaries, swelling of endothelial cells, and increase in mesangial cells.

FIG. 2. Case 13, fully developed picture. Glomerulus tremendously enlarged, very cellular and bloodless.

FIG. 3. Case 9, more advanced stage. An early epithelial crescent may be noted on the right.

FIG. 4. Case 11, late stage. Capillaries again patent, but contain few red blood cells.



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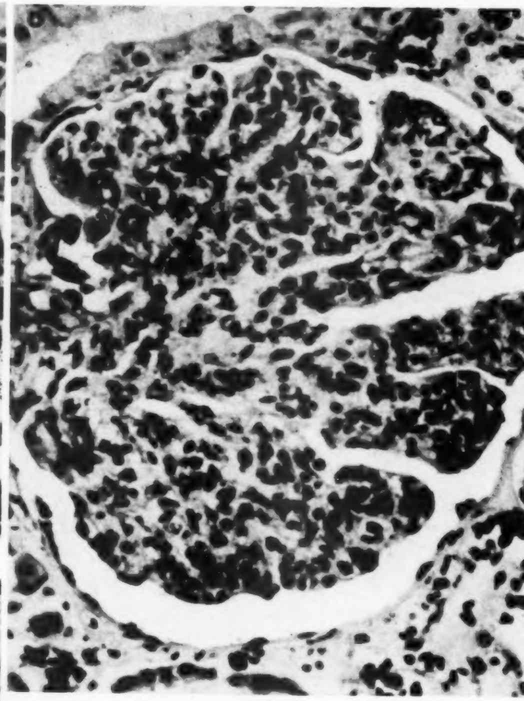

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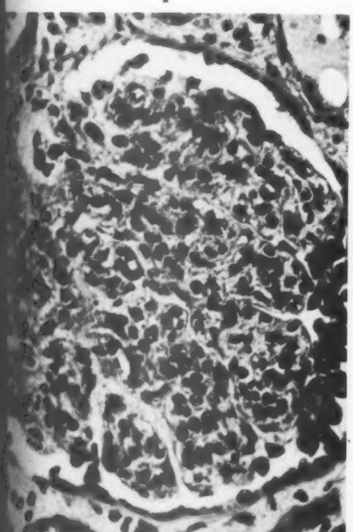

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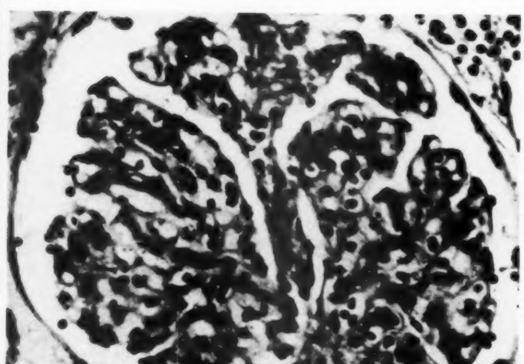

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Figs. 5 to 12. Glomerular lobule under high magnification. Sections were cut at a thickness of 0.5 μ. Periodic acid-Schiff's reagent and hematoxylin. $\times$ 1,500.

FIG. 5. Male, age 45. Normal glomerulus. Lobule with a central stalk (arrow) and a wreath of capillaries. Two nuclei can be seen in the mesangium.

FIG. 6. Male, age 39. Rheumatic heart disease. Lobule in shape of clover leaf, showing edema of mesangium. There is a faint membrane separating capillary lumen from mesangium, particularly in upper capillary.

FIG. 7. Same case as that from which Figure 1 was taken. Lobule showing edema and increased number of cells in mesangium. There is beginning separation of endothelial and epithelial basement membranes by edema fluid in capillary on the right.

FIG. 8. Case 4, marked widening of mesangium by edema and cellular exudate. Of note is the clear demarcation of capillary lumina from mesangium.

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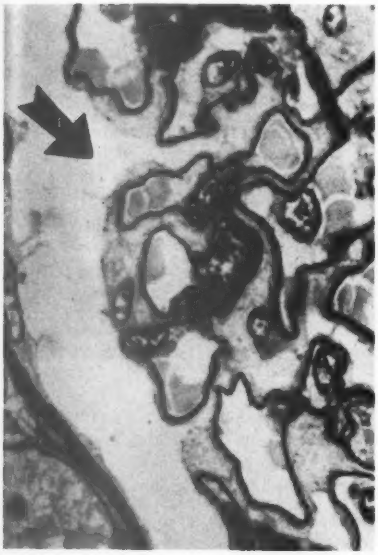

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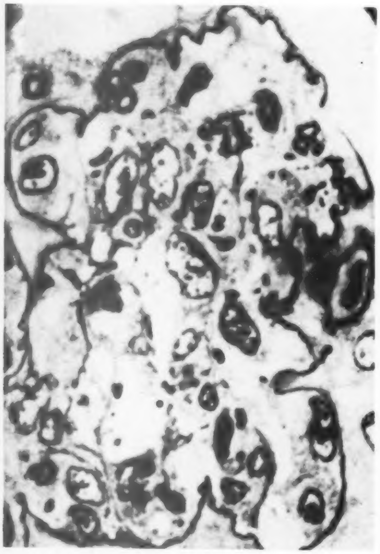
FIG. 9. Same case as that from which Figure 2 was taken. Tremendously enlarged lobule showing edema and marked proliferation of mononuclear cells. Complete loss of normal architecture.

FIG. 10. Same case as that from which Figure 3 was taken. Enlarged lobule showing edema, mononuclear cells, and a number of branching fibers in the mesangium. The capillary along the periphery is cut longitudinally. A distinct membrane separates the lumen from the mesangium.

FIG. 11. Same case as that from which Figure 10 was taken. Transverse section of peripheral lobule. Some of the capillaries are almost completely separated from the epithelial basement membrane by edema fluid and cells.

FIG. 12. Same case as that from which Figure 4 was taken. Lobule only slightly larger than normal. Capillaries patent but bloodless. Enlarged mesangium containing cells and many fibers.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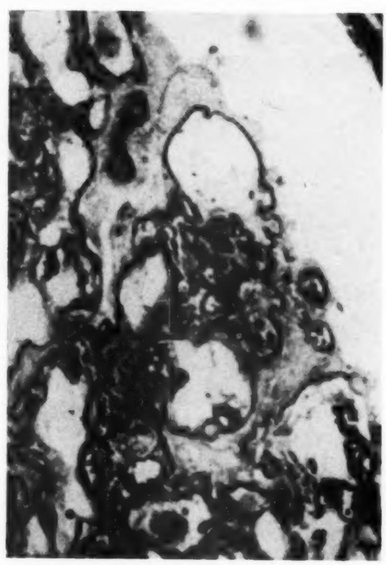
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STUDIES ON HEPATITIS IN HAMSTERS INFECTED WITH EQUINE ABORTION VIRUS

II. Changes in Protein, Nucleic Acid, and Weight of Isolated Hepatic Nuclei *

E. C. Bracken, Ph.D., and Charles C. Randall, M.D.†

From the Department of Microbiology, Vanderbilt University School of Medicine, Nashville, Tenn.

During infection of the hamster with equine abortion virus a series of progressive changes occur in the nuclei of the parenchymal cells of the liver.[1] In early stages inclusion material is not abundant, but from 9 to 12 hours the nuclei become filled with Feulgen-positive material. The LD_{50} titers of blood and liver reach a maximum between 9 and 12 hours and are concomitant with the development of changes in practically all the hepatic parenchymal cells. Other workers have investigated the possibility of chemical changes occurring in nuclei in which intranuclear inclusions appear. Johnson and Ackermann[2] reported that the nucleic acid content per cell of chick livers was found to be unaltered as a consequence of infection with herpes virus.

The consistently reproducible and rapidly lethal equine abortion virus hepatitis in hamsters, coupled with the demonstrated cellular changes, appears to offer an ideal experimental tool. The ensuing report will deal with certain unique chemical and physical changes in hepatic cells resulting from this infection.

Materials and Methods

The Virus, Experimental Animals, Inoculation, and Collection of Tissues

A description of this phase of the work has been documented.[1] The only variation was in the occasional use, as controls, of tissues from uninoculated normal hamsters, and tissues from animals inoculated with suspensions of normal liver.

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This report is from a dissertation submitted by Dr. Bracken in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Vanderbilt University.

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† Now at the Department of Microbiology, University of Mississippi, School of Medicine, Jackson, Miss.

Isolation of Nuclei of Hepatic Cells

Nuclei were isolated by the Dounce method,[3] employing homogenization in citric acid followed by a series of differential centrifugations. Ice-cold 0.01 M citric acid (final pH, 3.8 to 4.0) was used as the suspending medium throughout the isolation procedure. Portions of the citric acid solution were frozen prior to use in the depressions of spot plates, forming 1.0 cc. lens-shaped pieces. Approximately one fourth of the suspending medium used in the homogenization consisted of the frozen acid.

Wet mounts of the material were prepared on glass slides at intervals during the isolation procedures and examined immediately with a light microscope for clumping and for purity of the preparations. In addition to these unstained wet mounts, smears of final preparations were dried and fixed in 10 per cent neutral formalin, followed by staining with hematoxylin and eosin and by the Feulgen reaction. The smears stained by the Feulgen reaction were counterstained with aqueous light green. Both methods were used as an effective means of detecting the presence and extent of contamination by cytoplasmic constituents, either the intact cells or strands of cytoplasmic material adhering to nuclei. In all analyses reported, contamination by whole cells was judged to be insignificant. The term infected nuclei arbitrarily refers to hepatic nuclei of all types obtained from the isolation procedure.

Lyophilization and Weighing of Nuclei

Glass ampules of 5 ml. size were cleaned and dried, then weighed accurately to 0.1 mg. and approximated to 0.01 mg. Aliquots of the stock suspensions of isolated nuclei were pipetted into weighed ampules by means of a 2.0 ml. measuring pipette, the end of which had been drawn out to a fine tip. These aliquots consisted of 2.0, 3.0, and 4.0 ml. of the suspension of nuclei, and were prepared in duplicate, both as a safeguard against the consequences of a laboratory accident and as a means of comparison, one with the other, for accuracy of weighing and pipetting.

After the ampules had been filled, they were placed in test tubes, 25 by 150 mm. in size, shell frozen at –45° to –50° C., and lyophilized. Although the dried nuclei were found not to be demonstrably hygroscopic, they were weighed as quickly as possible upon removal from the lyophilizer. The recorded weights of the empty ampules were subtracted from the weights of the ampules containing the dried nuclei to

give the net weights of the nuclei. The weights of the citric acid present in the solutions also were subtracted from the weights of dried nuclei.

Enumeration of Nuclei

Counting was done by means of a hemacytometer and a minimum of 1,200 nuclei from each preparation was counted. Aliquots of stock suspensions of nuclei were diluted for counting 1:2.5, 1:5, or 1:10 in 0.01 M citric acid containing 0.01 per cent crystal violet. The counts were calculated on the basis of nuclei per ml. of the stock suspension, and the counts per ml. thus obtained were used to reduce weights of nuclei per ml. and weights of protein and nucleic acids per ml., to weights per nucleus.

Desoxyribonucleic Acid

Desoxyribonucleic acid (DNA) was estimated on the basis of content per nucleus. For that purpose, the diphenylamine reaction of Dische[4] was employed. Several procedures for the extraction of DNA from nuclei were tried and compared. The Dische reagents were observed to digest the nuclei in suspensions which were analyzed soon after preparation, and it was thought, therefore, that the DNA would be released and sufficient hydrolysis would take place during the 10 minutes of boiling to develop maximum color. However, the concentrations of DNA indicated by this procedure were almost uniformly 10 per cent less than the results obtained when nuclei had been suspended in 0.01 M citric acid or in water and stored under refrigeration for 24 to 48 hours. Evidently, considerable release and hydrolysis occurred under these conditions. Schneider's method[5] of extraction with trichloroacetic acid (TCA) was tried, and it was found that two successive extractions with 5 per cent TCA each at 90° C. for 15 minutes gave results in very close agreement with those obtained from nuclei stored in citric acid or in water for 24 to 48 hours. Single extractions were not sufficient; measurable quantities of DNA were found to be present in the second extracts. This method of two extractions in hot TCA was adopted for all experimentation involving nucleic acids.

Ribonucleic Acid

Ribonucleic acid (RNA), like DNA, was estimated on the basis of content per nucleus. Mejbaum's method,[6] employing the orcinol colorimetric reaction, was followed. A correction was applied for the color due to reaction with DNA. Two extractions in hot 5 per cent TCA, as described above, were used.

Proteins

A modification of the biuret method of Robinson and Hogden[7] was employed for the estimation of proteins. The principal modification of their procedure was the substitution of crystallized edestin for diluted rabbit serum as a standard. The results were expressed as milligram equivalents of edestin to protein per nucleus. Samples consisted of lyophilized residues from nuclei which had been extracted in TCA. This material was digested overnight in quantities of 3 per cent NaOH equal to the volumes of the original suspensions of nuclei from which the residue samples were derived.

Results

Desoxyribonucleic Acid

Investigations were made to determine whether or not any changes in content of DNA in nuclei existed during the course of infection, from the time of inoculation to fatal termination. Hamsters were inoculated and representative numbers of them were killed at 3, 6, 9, 12, and 15 hours after inoculation. Nuclei were isolated from cells of livers of these animals and analyzed for DNA. This experiment was repeated with material from three separate passages of the virus (P-110, P-118, and P-124) and from one series of controls inoculated with suspensions of normal liver and sacrificed at the intervals stated above. Several groups of normal uninoculated control animals and groups of animals in the terminal stages of infection also were included.

As indicated in Table I, no significant changes in amounts of DNA

Table I

Average DNA per Nucleus (mg. $\times 10^{-9}$) of Infected and Uninfected Cells

	Uninoculated controls	Inoculated controls	Infected 3–9 hrs.	Infected 12–15 hrs.	Infected terminally
	10.30	10.40	10.42	10.26	10.13
	10.50	10.57	10.46	10.05	10.20
	10.54	10.69	10.62	10.61	10.44
	10.79	10.80	10.66	11.05	10.80
	11.00	11.14	11.06	11.15	11.10
			11.20		11,40
Av.	10.63 ± 0.12	10.72 ± 0.12	10.74 ± 0.13	10.62 ± 0.17	10.68±0.21

were observed under any of the experimental conditions. The average DNA content per nucleus was computed to be 10.70×10^{-9} mg. The DNA determinations used in computing the average are listed in

Table I according to the category of the nuclei; i.e., uninoculated controls, inoculated controls, early infections (3 to 9 hours), late infections (12 to 15 hours), and terminal infections. It can be seen from these data that there are no significant differences in the DNA among any of these categories of nuclei. The factor of polyploidy was not taken into consideration, and the results represent an average value per nucleus.

The possibility existed that nuclei of infected cells were more fragile than those of the controls, which would lead to erroneous results if they were selectively destroyed in the homogenization process. This possibility was evaluated by testing the supernatants obtained after the initial homogenization and centrifugation of livers for the presence of DNA. No measurable quantities were found in any of the samples selected at random for analysis. Suspensions of nuclei in distilled water and in 0.01 M citric acid were stored for 1 to 3 weeks in a refrigerator, after which analyses of supernatants for DNA were made. The DNA of the supernatants amounted to a maximum of 0.5 per cent of that contained in the whole suspension.

Weights of Nuclei

It was noted in preliminary experimentation that the livers from infected animals weighed as much as 30 to 35 per cent more than

TABLE II

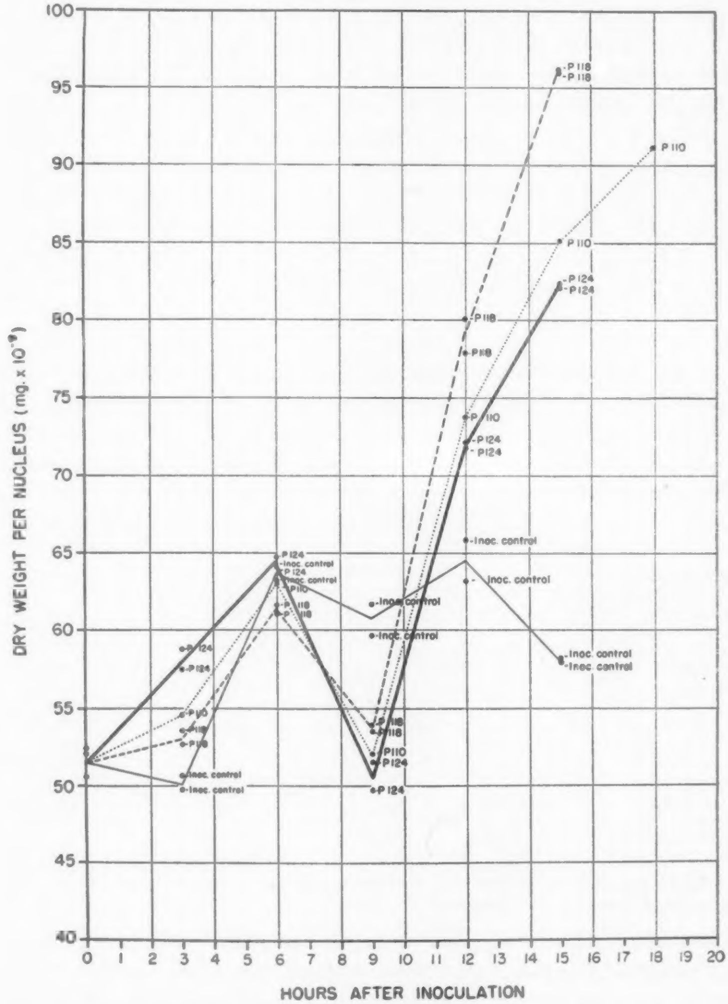
*Comparisons of Dry Weights of Nuclei of Cells from Infected and Uninfected Hamsters**

Nuclei from	Dry weights of nuclei (mg. $\times 10^{-9}$/nucleus)	Nuclei from	Dry weights of nuclei (mg. $\times 10^{-9}$/nucleus)
Uninoculated control	51.00	P-110 (18 hrs.)	91.11
Uninoculated control	52.44	P-115 (terminal)	60.82
Uninoculated control	50.46	P-117 (terminal)	77.60
Uninoculated control	51.99 and 52.31	P-118 (3 hrs.)	52.66 and 53.52
Inoculated control (3 hrs.)	49.70 and 50.55	P-118 (6 hrs.)	61.06 and 61.96
Inoculated control (6 hrs.)	63.10 and 64.17	P-118 (9 hrs.)	53.52 and 53.81
Inoculated control (9 hrs.)	59.57 and 61.62	P-118 (12 hrs.)	77.78 and 80.01
Inoculated control (12 hrs.)	63.12 and 65.80	P-118 (15 hrs.)	95.94 and 96.04
Inoculated control (15 hrs.)	57.98 and 58.05	P-119 (terminal)	97.53 and 98.95
P-109 (terminal)	78.00	P-124 (3 hrs.)	57.40 and 58.70
P-110 (3 hrs.)	54.40	P-124 (6 hrs.)	64.05 and 64.69
P-110 (6 hrs.)	62.95	P-124 (9 hrs.)	49.74 and 51.52
P-110 (9 hrs.)	52.05	P-124 (12 hrs.)	71.74 and 72.02
P-110 (12 hrs.)	73.67	P-124 (15 hrs.)	82.01 and 82.25
P-110 (15 hrs.)	85.03		

* Weights derived from single and/or duplicate aliquots of dried nuclei obtained from pooled livers of either 4 or 5 hamsters.

normal livers. The possibility that this was due to the extra day or two of age of the infected animals at death was eliminated by killing the control animals at the same time that the infected ones died. An-

other possibility was that the differences in weights were due to an edematous condition of the livers of infected animals, although this factor was not experimentally evaluated. A third possibility was that the weight differences resided in the nuclei as a direct result of the infection. In order to test this last supposition, nuclei were isolated by the usual procedure, and then were lyophilized and weighed. As shown in Table II, weights of dried control nuclei were consistently less than



Text-figure 1. Dry weights of nuclei at time intervals after inoculation.

those from terminally infected animals, and the weights formed reproducible and characteristic curves when weighed samples from sequential series were plotted graphically as weight versus time of infection. Text-figure 1 shows characteristic curves resulting from graphic representation of the weights of nuclei from liver cells of animals from P-110, P-118, and P-124 at 3-hour intervals, as well as a series of controls inoculated with normal liver. The weights of the nuclei of cells from uninoculated control animals are indicated in the graph at 0 hours. The weights shown in Table II were derived from single aliquots of nuclei isolated from the pooled livers of four or five hamsters, except that when sufficient material was available, duplicate aliquots of the same materials were prepared and weighed. These duplicates are indicated in Table II and are individually plotted in Text-figure 1.

It will be noted (Text-fig. 1) that the weights of the inoculated control nuclei increased from those of the normal controls until 6 hours after inoculation, but did not change materially thereafter. A similar increase in the weights of the infected nuclei was noted between 0 and 6 hours, but the weights decreased between 6 and 9 hours, and a continuous and marked increase took place between 9 and 15 hours. If the changes during the early period after inoculation (to 9 hours) have statistical significance, it may be conjectured that the increases between 0 and 6 hours were due to absorption and concentration in the liver of the foreign material contained in the inoculum. However, it is difficult to interpret on this basis the subsequent decrease (6 to 9 hours) in the weights of the infected nuclei, especially in view of the fact that the infected animals had been injected with the same quantity of liver suspension as the inoculated controls. The increases observed during the period of 9 to 15 hours were of such magnitude that they are obviously significant.

Proteins

The next experiments were undertaken to determine more specifically the substances in the nuclei which were contributing to the increase in weight. It will be recalled that DNA had been found to be essentially constant. Analyses were made for protein in nuclei of hepatic cells from uninoculated control animals, from P-118 and P-124 at 3-hour intervals, and from control animals inoculated with normal liver and killed at intervals. The results, indicated in Table III and Text-figure 2, were expressed as milligram equivalents of edestin per nucleus. A comparison of Text-figures 1 and 2 shows that the weights of nuclei and of nuclear proteins closely parallel each other at comparable intervals.

Ribonucleic Acid

Inasmuch as the foregoing experiments indicated that protein accumulated in the nuclei of hepatic cells as infection with equine abortion virus (EAV) progressed, it seemed possible that similar and concomitant quantitative changes in RNA might take place. Accordingly, analyses for RNA were made from nuclei of hepatic cells from three separate groups of uninoculated control animals, from animals in the terminal stages of infection (EAV P-119), and from two groups of inoculated animals (EAV P-118 and EAV P-134) killed at 3-hour intervals.

TABLE III

Comparisons of Content of Nuclear Proteins from Infected and Uninfected Hamsters*

Nuclei from	Protein† (mg. × 10^{-9}/nucleus)
Control (normal liver)	32.0
P-124 (3 hrs.)	34.7
P-124 (6 hrs.)	39.6
P-124 (9 hrs.)	30.1
P-124 (12 hrs.)	43.2
P-124 (15 hrs.)	49.9
P-118 (3 hrs.)	34.2
P-118 (6 hrs.)	39.5
P-118 (9 hrs.)	35.1
P-118 (12 hrs.)	52.9
P-118 (15 hrs.)	58.0
Inoculated control (3 hrs.)	30.2
Inoculated control (6 hrs.)	40.6
Inoculated control (9 hrs.)	36.4
Inoculated control (12 hrs.)	38.2
Inoculated control (15 hrs.)	35.0
P-117 (terminal)	46.0
P-119 (terminal)	61.0

* Weight equivalents of edestin.

† Each determination of protein represents a single analysis of nuclei isolated from pooled livers of either 4 or 5 hamsters.

TABLE IV

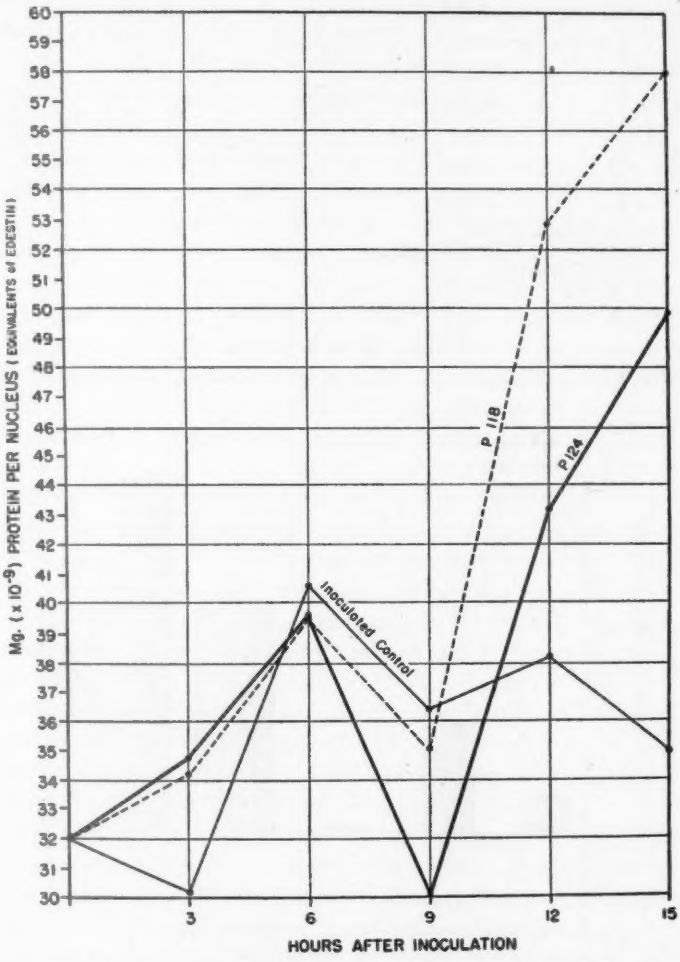
*The Ribonucleic Acid Content of Hepatic Cell Nuclei from Hamsters at Different Stages of Infection with Equine Abortion Virus**

Nuclei from	RNA per nucleus (mg. × 10^{-9})
Uninoculated control	2.98
Uninoculated control	2.75
Uninoculated control	2.70
P-118 (3 hrs.)	2.58
P-118 (6 hrs.)	2.83
P-118 (9 hrs.)	2.77
P-118 (12 hrs.)	4.32
P-118 (15 hrs.)	4.30
P-134 (3 hrs.)	2.88
P-134 (6 hrs.)	2.80
P-134 (9 hrs.)	2.77
P-134 (12 hrs.)	3.70
P-134 (15 hrs.)	4.54
P-119 (terminal)	3.53

* Each determination represents a single analysis of nuclei isolated from pooled livers of either 4 or 5 hamsters.

The results, which are summarized in Table IV and Text-figure 3, indicated very little change in RNA concentration until after the infection had progressed to 9 hours. However, the RNA increased greatly between 9 and 15 hours, amounting to more than a 60 per cent increase over the controls. Some variations were found, particularly at 12 hours, between the RNA concentrations in the two virus passage materials used. At 12 hours the RNA from P-118 considerably exceeded that from P-134, although no further increase occurred in the

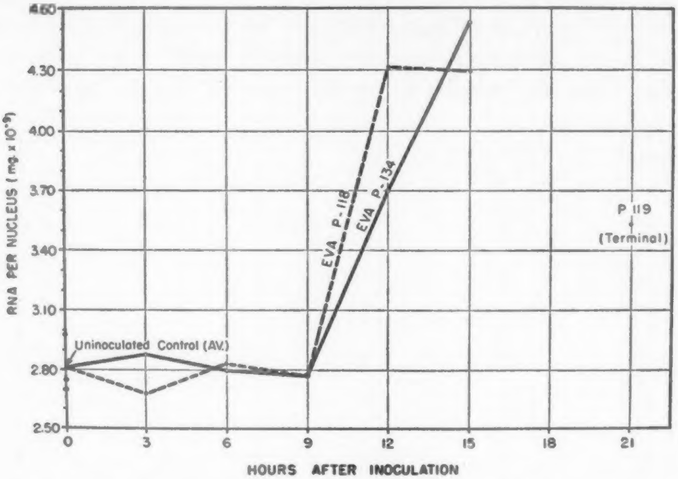
material from P-118 at 15 hours. It should be mentioned that the virus was being passed weekly at the time of passage 118, but bi-weekly for passage 134. Thus, the infection may have progressed somewhat more rapidly in the earlier passage, and may account for the higher level of RNA found in this passage material at 12 hours. The RNA content of the hepatic nuclei from animals in the terminal stages of infection (EAV P-119) decreased markedly from the levels at 15 hours. The animals which comprised this passage material died at intervals be-



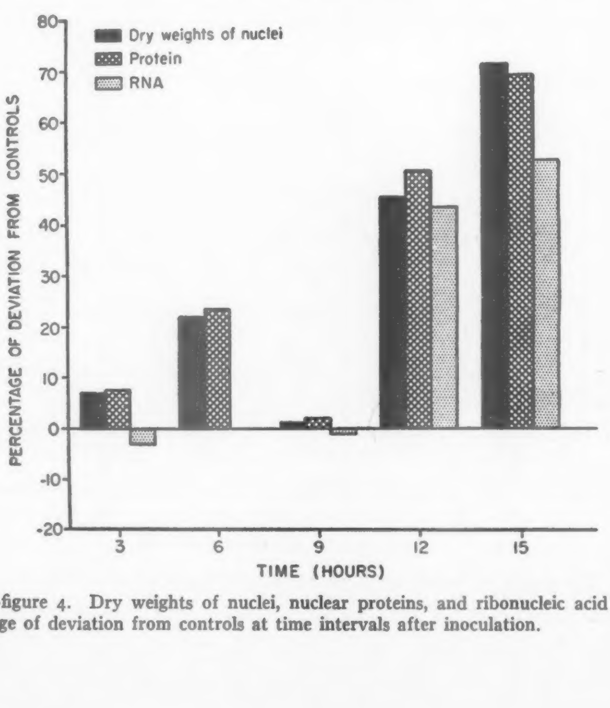
Text-figure 2. Protein per nucleus at time intervals after inoculation.

tween 18 and 24 hours after inoculation, and the RNA concentration, therefore, was plotted in Text-figure 3 arbitrarily at 21 hours.

A comparison of Table II and Text-figure 1 and Table III and Text-figure 2 with Table IV and Text-figure 3 indicates that variations in



Text-figure 3. Ribonucleic acid per nucleus at time intervals after inoculation.



Text-figure 4. Dry weights of nuclei, nuclear proteins, and ribonucleic acid (RNA). Percentage of deviation from controls at time intervals after inoculation.

quantities of RNA per nucleus approximately parallel the variations in total weight per nucleus and quantities of protein per nucleus as the infection progressed. These data also are shown in Text-figure 4 as percentages of change from normal controls.

Discussion

In this study the constancy of nuclear DNA in both control and infected nuclei has been established. It will be recalled that according to the preceding paper[1] each nucleus (usually) became filled with a markedly Feulgen-positive inclusion. The question arises as to the source of the material. Since there is no attendant increase in DNA, is there a redistribution of this nuclear component, or is synthesis balanced by release from the nucleus? Consideration may be given to the number and distribution of viral elementary bodies in other virus diseases in which there are intranuclear inclusions. Electron microscopy of infected nuclei[8,9] indicates that the supposed elementary bodies are not uniformly arranged. Nothing is known concerning the structure and chemical composition of the elementary particles of equine abortion virus, but it is not likely that the numerical and spatial arrangement would be such that an inclusion would be formed. It has been suggested[2] that the gross methods of analysis currently available would not enable one to detect the small differences in total DNA which might be directly attributable to the presence of mature viral particles.

The finding, in this study, of no significant changes in DNA content of infected nuclei is consistent with the observations of others who have compared the nucleic acids of normal cells with those from animals under conditions of irradiation, malignancy, or treatment with toxic chemicals.[10-12] Griffin and coworkers[13] found, however, an increased content of DNA in hepatic cells of rats during azo-dye carcinogenesis.

It should be mentioned that apparently there is no change in ploidy of the hepatic cells under conditions of infection with equine abortion virus, inasmuch as changes in DNA per nucleus could have been expected if there was a drastic shift in the ploidy classes.

No attempts were made in the course of this study to obtain and compare enumerations and weights of the total complement of cells in entire dried livers. However, it seemed likely that the observed 30 to 35 per cent increase in the total wet weights of infected livers over the weights of control livers could be accounted for, at least in part, by increase in weight of the nuclei of infected cells rather than by an increase in total number of cells. This view appears to have been justified

largely by our finding of greatly increased weights of the nuclei of cells from infected animals. In turn, the increase in weight of nuclei was found to be due chiefly, if not entirely, to increases in nuclear proteins and RNA. Ackermann and Francis[14] attributed their observations of increased nucleic acids to an increase in the number of cells per unit weight of tissue.

The finding, in the present study, of considerable increases in nuclear RNA in cells characterized by the presence of intranuclear inclusion bodies appears to be unique, although several investigators[10,12,15] have reported that changes in RNA concentrations occur in nuclei of cells which were abnormal by reason of malignancy, irradiation, or treatment with drugs. Laird[10] has reported, in studies of nuclei from hepatic cells of rats treated with thioacetamide, that not only the RNA increased, but that nuclear proteins increased to about 2.5 times the normal.

It is of interest to note (Text-fig. 4) the tendency toward linear increases, except at 9 hours, of weights of nuclei, proteins, and RNA. Obviously, the greatest changes occur between 9 and 12 hours. It will be recalled[1] that the development of inclusions and the virus titer also reach a maximum during this same interval.

There is as yet no evidence to explain the mechanism of the increase of nuclear proteins and RNA. In the realm of speculation, these changes may be considered to be due to accumulation of material from actual elementary bodies, genetic alterations of the host cells by the virus, or to changes in the properties of the nuclear membranes and/or the proteins of the host cells. The magnitude of increases of proteins and RNA would require the presence of vast numbers of elementary bodies in each nucleus if the increases were due to the virus particles themselves. It is exceedingly unlikely that such conditions in virus-host cell relationships would be found. On the other hand, it is entirely possible that the virus may direct new enzymatic activities of the host cells, probably by substitution for, or addition to, host genes. It also would seem possible that the virus in some manner may interfere with the functioning of the nuclear membrane. Although electron microscopy of nuclei infected with herpes simplex[9] indicates that the nuclear membranes may be disrupted, an otherwise plausible explanation of the manner by which such interference might occur seems to be afforded by the observation[1] of accumulations of chromatin at the periphery of nuclei infected with the equine abortion virus. It is possible that this marginated material could constitute a mechanical blockage or "plugging" of the nuclear membrane. It might be presumed that materials, such as RNA and nuclear proteins, are prevented from diffusing across

the membrane into the cytoplasm and thus tend to accumulate in the nucleus. If this is true, interesting supportive studies might be made to determine whether or not reciprocal diminutions of cytoplasmic RNA and proteins could be detected. At the least, these suggestions are highly compatible with the present evidence[16] that the cell nucleus plays a very important rôle in the metabolism of RNA and nucleotide.

Whether or not any changes occur in the properties of the nuclear membranes, there may be changes in the nuclear proteins which would prevent their free movement within the cell, such as the nuclear proteins being rendered insoluble or denatured by the influence of the virus.

If the increased nuclear proteins are the result of accelerated protein synthesis, and since protein synthesis is considered to be intimately associated with RNA concentrations, it is not surprising that greatly increased production of protein takes place in the infected cell under conditions of increased RNA.

The results of the present investigations indicate that hepatitis in hamsters induced by equine abortion virus comprises an ideal research system for studies of animal virus-host cell relationships. It also may lead to a better understanding of several aspects of the mechanism of cellular metabolism.

Summary

A sequential study is reported of changes occurring in hamsters infected with equine abortion virus. Hepatic cell nuclei were isolated in citric acid, enumerated, lyophilized, weighed, and analyzed. Desoxyribonucleic acid (DNA), ribonucleic acid (RNA), nuclear proteins, and dry weights of nuclei were expressed in units of mg. $\times 10^{-9}$ per nucleus. The average weight of uninoculated control nuclei was 52. The inoculated controls increased in weight between 0 and 6 hours, but did not change appreciably thereafter. During the period from 0 to 6 hours, changes in the infected nuclei paralleled those of the inoculated controls. The possible significance of the increase in weight of nuclei during the early period is discussed. By contrast, it has been shown that the infected nuclei between 9 and 15 hours increased in weight to the significant figure of 90.

Weights of proteins closely paralleled weights of nuclei at corresponding intervals. DNA did not change significantly at any intervals of infection and averaged 10.70 both in control and infected nuclei. RNA did not vary appreciably from the control figure of 2.81 (average) until after 9 hours. Between 9 and 15 hours the amount per nucleus rose to an average of 4.42.

The findings of increased weight of nuclei and increased RNA and proteins with unchanged DNA values resulting from an animal virus infection appear to be unique. Further work is in progress in an attempt to elucidate these phenomena.

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HISTOPATHOLOGIC REACTIONS OF THE YOLK SAC TISSUE OF EMBRYONATED HEN'S EGGS TO COCCIDIOIDES IMMITIS *

R. A. VOGEL, Ph.D.; R. J. PEACE, M.D., and M. T. KOGER

From the Research and Clinical Laboratories of the Veterans Administration Hospital, and the Departments of Bacteriology and of Pathology, Emory University School of Medicine, Atlanta, Ga.

Attempts to cultivate the spherule or parasitic phase of *Coccidioides immitis* in the embryonated hen's egg have met with varying degrees of success.[1-5] Previous investigations showed that inoculation of the yolk sac with heavy suspensions of arthrospores led to the development of large numbers of both endosporulating and immature spherules. The structure and development of this phase of the fungus were clearly visualized in direct microscopic preparations of yolk fluid. Material obtained by culture of inoculated yolk sac has been used to immunize guinea pigs against coccidioidal infection[6] and as a source of complement fixing antigen to detect antibodies in rabbit antisera.[7] Notwithstanding this successful use of the technique, many phases of egg cultivation of this fungus remain to be elucidated. For reasons which are obscure at present, mortality of eggs after infection with the fungus has been totally unpredictable. With this problem in mind, a histopathologic study was made to determine whether the fungus after injection into the yolk sac remained suspended in the yolk menstruum or actually invaded the tissue of the yolk sac.

MATERIALS AND METHODS

The open window method for inoculating the yolk sac was employed. An inoculum was prepared from 3-month-old Sabouraud's agar cultures of *C. immitis*.[4] The arthrospores were suspended in 0.5 per cent "Tween 80"-saline solution and washed in brain-heart infusion broth. The turbidity of the inoculum approached that of a no. 9 MacFarland nephelometer tube. The 13-day-old egg was most suitable for high survival of embryos and was used in obtaining the material considered in this report. After inoculation with approximately 0.5 ml. of the fungal suspension, the eggs were incubated at 37° C. They were opened at various intervals and preparations of yolk fluid examined microscopically. Small pieces of tissues from the yolk sac were carefully mounted on paper and fixed in 10 per cent formalin. Sections cut

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from these preparations were stained with hematoxylin and eosin, periodic acid-Schiff, and Giemsa stains.

Results

Large, vacuolated spherules were observed in direct microscopic preparations of yolk fluid from infected eggs studied after 3 days. They ranged in size from 30 to 80 μ and had an orange tint under tungsten illumination. More spherules were observed in material scraped from the inner folds of the yolk sac, possibly because the large maturing spherule sediments in the stationary egg and comes to rest adjacent to the yolk sac. After 4 to 5 days of incubation, the vacuolated spherule had been largely replaced by clusters of endospores which were difficult to discern from fat droplets except when seen in clumps near the casing of an empty spherule and subsequently noted to germinate in a Van Tieghem mount.[8]

Histopathologic Examination

After 5 to 6 days of incubation, organisms were readily identified and easily distinguished from artifacts (Figs. 1 and 2). A prominent feature was direct invasion of tissue, with response of two types to the injury. The first and more prominent was a disseminated, chronic inflammatory reaction of endothelial and reticulohistiocytic elements of the primitive blood vascular system. This was miliary in nature, confined to peripheral tissue fronds, and took the form of granulomatous angiitis. Granulomas were characterized by multinucleated giant cells of foreign body type that sometimes contained phagocytized spherules (Figs. 3 and 4). They also were set in compact aggregations of poorly differentiated littoral cells and fixed histiocytes (Figs. 5 and 6). Karyorrhectic primitive polymorphonuclear leukocytes were encountered occasionally in large granulomas. Often, recently deposited spherules were found free in the lumina of capillaries and had not yet provoked an inflammatory response (Fig. 7). The granulomas were continuous with and involved endothelial cells of blood vessels in their formation, the mechanism of which was obviously one of embolization of terminal capillary loops by coccidioidal organisms that had gained access to the circulating blood of the embryonal membrane.

Less typical but not characteristic of this response to injury were segments of blood vessels showing endovasculitis and perivasculitis with few or no identifiable organisms. These lesions were oriented to

blood vessels but not of obvious mechanical origin, being more loosely knit and of such a nature as to suggest a humoral or chemical etiology. Liquefaction necrosis of fixed and free tissue elements was a definite but not a prominent feature of some granulomas. Occasionally the lumina of capillaries involved in the inflammatory process were filled with strands of fibrin and an amorphous proteinogenous coagulum. Mycelium was seldom encountered in granulomas of either type, but its presence was associated with necrosis and an increase in the number of polymorphonuclear leukocytes.

Myriads of spherules and some mycelial elements were suspended in the yolk menstruum. Where these were in apposition to peripheral vesicular cells of the tissue fronds there was minimal liquefaction necrosis and minimal destruction of the margins of cells without reticulohistiocytic response as in the granulomas (Figs. 3 and 7). In areas where this reaction was best developed there was collapse of the structural pattern of the usually well defined tissue fronds seen in uninfected eggs.

Discussion

The survival of embryos for as long as 5 to 6 days after yolk sac infection with the saprophytic phase of *C. immitis* is of primary interest for harvesting spherule material since the latter only develops in the living egg. Burke, Salvin, and Gerloff[3] have reported that embryo survival increases with the age of the egg. Under the experimental conditions cited in this report it has not been possible to establish decisively that age is a critical factor in survival. Unpublished findings support the fact that in using 9 to 13 day-old eggs, the 10-day eggs often have a higher survival rate of embryos after infection than the older 11-day eggs. The factors contributing to these unusual findings remain obscure. However, they may be found in the many variables involved in handling the eggs prior to infection or to variability in the trauma caused by penetration of the highly vascular chorioallantoic and yolk sac membranes during inoculation.

Moore[9] described the pathologic features of a coccidioidal lesion which developed on the chorioallantoic membrane of developing chicks after direct application of a suspension of arthrospores. The membrane showed little response to the organism. Newcomer *et al.*[4] recently reported failure in their attempts to demonstrate the spherule phase in the extra-embryonic membranes. Their material was obtained from eggs inoculated in the yolk sac and allantoic cavity. The granulomatous angiitis and foreign body giant cell response in the tissues of the yolk sac which were observed in our material are similar to those described

on the chorioallantoic membrane by Monteiro *et al.*[10] in their work with *Paracoccidioides brasiliensis*. It is undeniable evidence that the organisms are capable of invading the tissues of the yolk sac and through involvement of the blood vessels ensure a hematogenous spread of the organism. The relationship of the lesions to the lack of survival of embryos cannot be evaluated at this time since too few eggs have been studied; and dissemination of the fungus throughout the body of the chick has not yet been observed.

Summary

The yolk sac of embryonated hen's eggs, 13 days old, was inoculated with the arthrospores or saprophytic stage of *Coccidioides immitis*. The spherule stage developed after 72 hours of incubation and was demonstrated in the yolk menstruum and in the tissues of the yolk sac. There was direct invasion of the tissues of the yolk sac by the fungus, characterized histologically by a granulomatous reaction which involved the primordial vascular elements.

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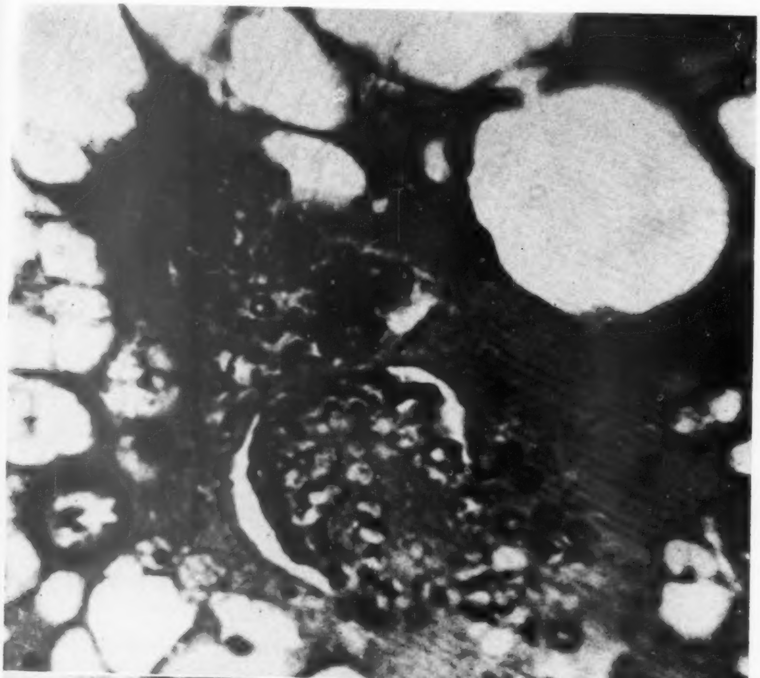
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIGS. 1 and 2. *Coccidioides immitis* in infected tissue of yolk sac, showing the large endosporulating spherule phase of the fungus characteristically seen in eggs incubated for 4 days. Figure 1, periodic acid-Schiff stain. × 640. Figure 2, hematoxylin and eosin stain. × 640.



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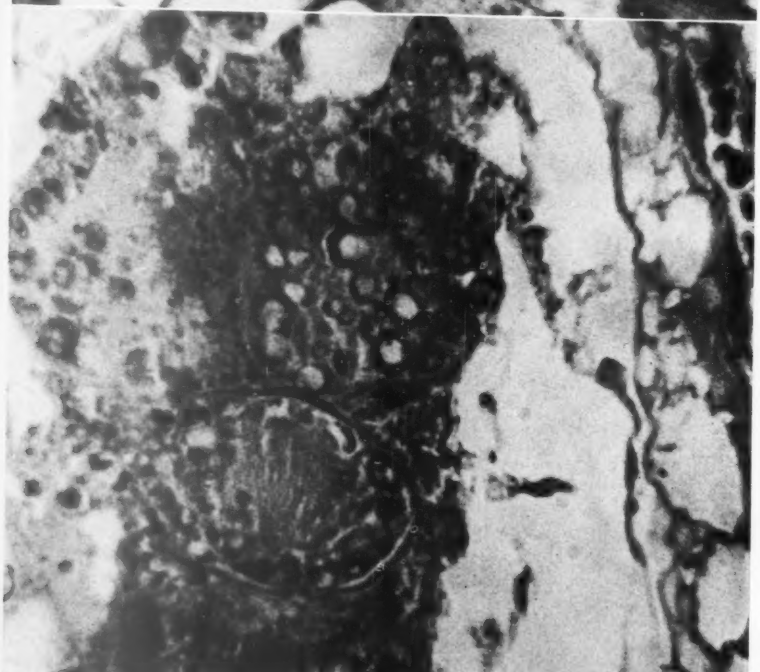

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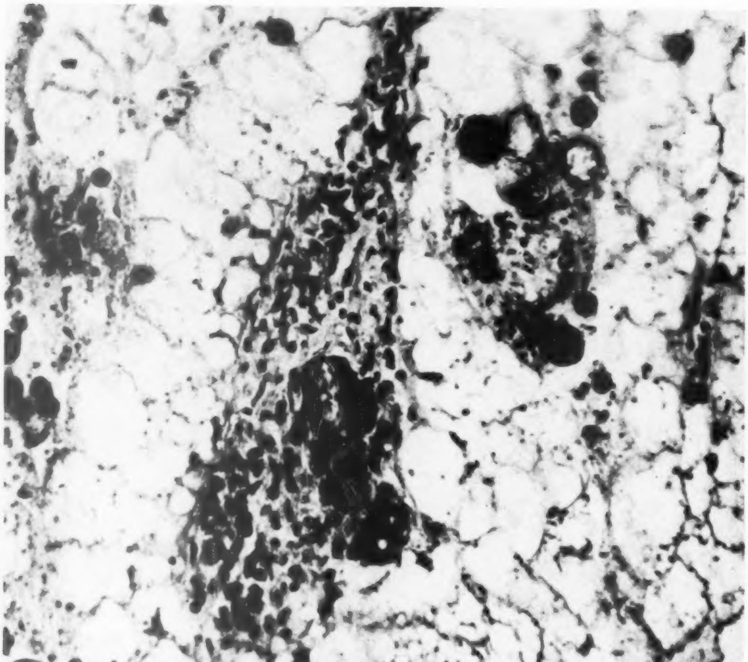
FIG. 3. Granulomatous angiitis in tissue fronds of an embryonated egg infected with *C. immitis*. Of note is the multinucleated foreign-body giant cell containing a large phagocytized spherule and lying alongside a capillary. Above and to the right of the granuloma, organisms are lying free in the yolk menstruum in a space between fronds. One organism shows definite endosporulation. Tissue in apposition to the organisms showed minimal liquefaction necrosis, blurring of cell outline, and loss of chromatic properties. Hematoxylin and eosin stain. × 420.

FIG. 4. Granulomatous angiitis in tissue fronds of an embryonated egg infected with *C. immitis*. Several multinucleated giant cells containing phagocytized spherules are arranged in granulomatous configuration and encroach upon a central capillary. Hematoxylin and eosin stain. × 420.



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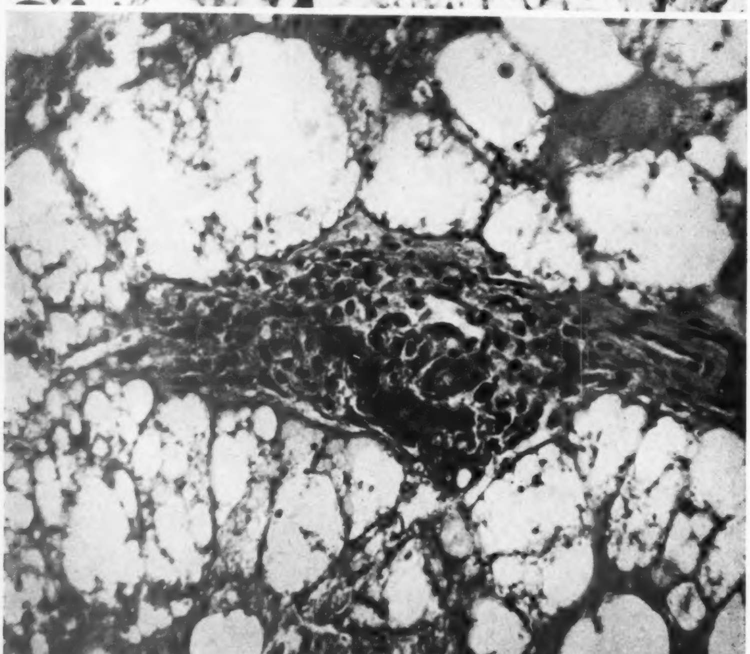

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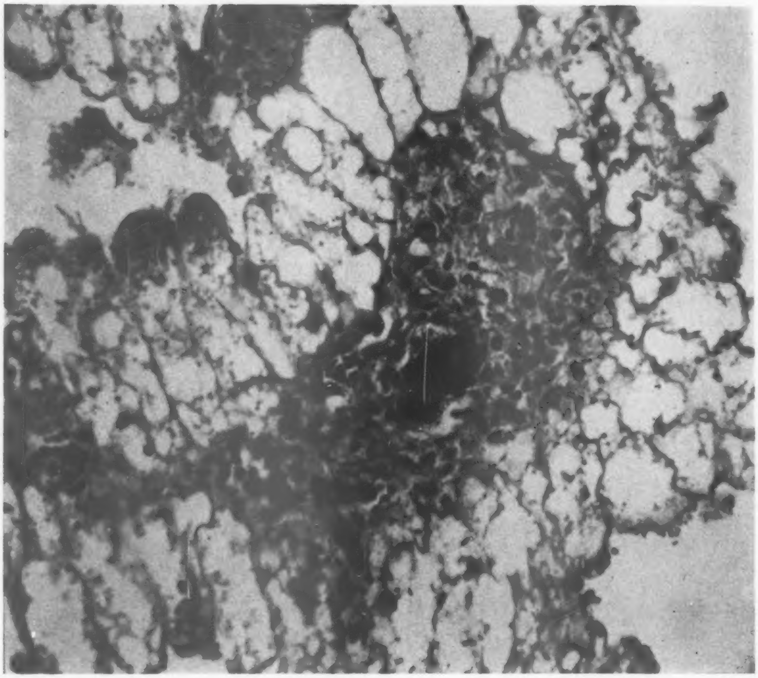
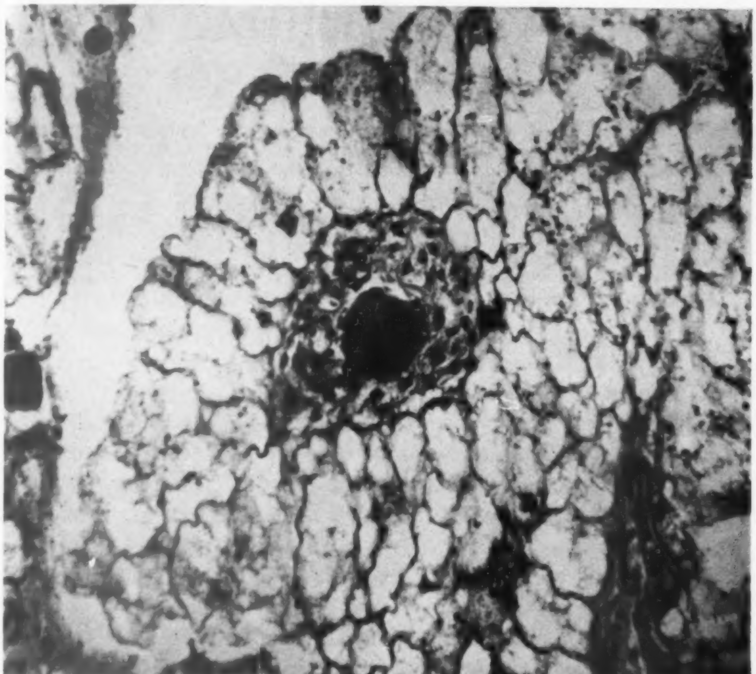


FIGS. 5 and 6. Granulomatous angiitis in tissue fronds of an embryonated egg infected with *C. immitis*. Some granulomas were characterized by well developed giant cells not containing organisms. Hematoxylin and eosin stain. × 420.

FIG. 7. Embryonated egg infected with *C. immitis*. On the right a large spherule represents an embolus in a central capillary of a tissue frond. On the left organisms lie free in the yolk menstruum. Hematoxylin and eosin stain. × 420.



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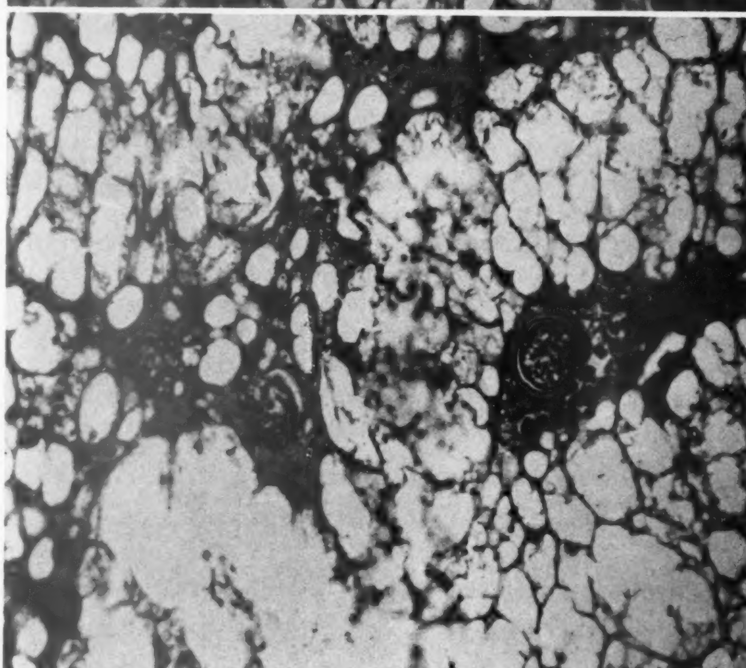

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